Self-association of dermatan sulphate proteoglycans from bovine sclera

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1. Two proteodermatan sulphate fractions (I and II) from bovine sclera were studied by gel chromatography, light-scattering and ultracentrifugation under various conditions. 2. Gel chromatography of proteoglycans in the absence or presence of hyaluronate was performed under associative conditions. No effect on the elution profile was noted. 3. Ultracentrifugation experiments (sedimentation-velocity and sedimentation-equilibrium) with proteoglycan I and II in 6 M-guanidine hydrochloride gave molecular weights ($\bar{M}_w$) of 160 000–220 000 and 70 000–100 000 respectively. As the protein contents were 45% and 60% respectively, it may be calculated that proteoglycan I contained four to five side chains, whereas proteoglycan II contained one or two. Sedimentation-equilibrium runs performed in 0.15 M-NaCl gave an apparent molecular weight ($\bar{M}_w$) of 500 000–800 000 for proteoglycan I and 90 000–110 000 for proteoglycan II. 4. In light-scattering experiments both proteoglycans I and II yielded high particle weights in 0.15 M-NaCl (3.1×10^6 and 3.4×10^6 daltons respectively). In the presence of 6 M-guanidine hydrochloride the molecular weights decreased to 410 000 and 130 000 respectively. The particle weights in 0.15 M-NaCl were not altered by the addition of hyaluronate or hyaluronate oligosaccharides. 5. The dermatan sulphate side chains of scleral proteoglycans (L-iduronate/D-glucuronate ratio 7:13) gave a particle weight of 100 000 daltons in 0.15 M-NaCl. In 1.00 M-KCl/0.02 M-EDTA the molecular weight was 24 000. Addition of free scleral dermatan sulphate chains to a solution of proteoglycan II promoted further multimerization of the macromolecule.

Proteoglycans composed of several glycosaminoglycan side chains linked to a protein core are present in most connective tissues (for a review see Muir & Hardingham, 1975). Cartilage proteoglycans can form multimolecular aggregates in which many proteoglycan molecules are bound to a chain of hyaluronate (Hardingham & Muir, 1972, 1973, 1974; Hascall & Heinegård, 1974a,b). Proteoglycans containing dermatan sulphate have been isolated from heart valves, skin, tendon and sclera (Toole & Lowther, 1965, 1968; Öbrink, 1972; Anderson, 1975; Damle *et al.*, 1979; Cöster & Fransson, 1981) as well as arterial wall (Ehrlich *et al.*, 1975; Eisenstein *et al.*, 1975; Radhakrishnamurthy *et al.*, 1977). The proteoglycans of arterial wall are generally of higher buoyant density and higher carbohydrate content than those of the other tissues.

The proteoglycans of bovine sclera have been separated by gel chromatography into two fractions

with different molecular sizes and protein/dermatan sulphate ratios (Cöster & Fransson, 1981). The high-molecular-weight proteoglycan I appeared to aggregate in 0.5 M-sodium acetate, whereas in 4 M-guanidine hydrochloride association was abolished. Dermatan sulphate chains themselves are able to self-associate (Fransson, 1976; Fransson & Cöster, 1979; Fransson *et al.*, 1979), and it was of interest to study the aggregation of scleral proteoglycans. Attempts were made to test whether multimerization of proteodermatan sulphates was mediated by hyaluronate, as in cartilage, by the dermatan sulphate side chains or by other means.

Experimental

Materials and methods

High-molecular-weight sodium hyaluronate was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Low-molecular-weight ^3H -labelled hyal-

uronate was prepared from fibroblasts (Sjöberg *et al.*, 1979). Hyaluronate oligosaccharides (deca-saccharides or larger) were prepared as described by Hascall & Heinegård (1974b).

Proteoglycans from bovine sclera were extracted with 4 M-guanidine hydrochloride in the presence of proteinase inhibitors and purified by ion-exchange chromatography and density-gradient centrifugation (Method A; Cöster & Fransson, 1981). In addition, a somewhat modified procedure (Method B) was employed. In this case the tissue extract was adjusted to a density of 1.30 g/ml by addition of solid CsCl and centrifuged (Cöster & Fransson, 1981). The bottom fractions ($\rho = 1.31\text{--}1.40$ g/ml), comprising approx. 80% of the total uronic acid content, were pooled and the material was re-centrifuged by using a starting density of 1.40 g/ml. In this case fractions with density >1.42 g/ml were pooled. In both cases (A and B) proteoglycans were subfractionated (into fractions I and II) by gel chromatography on Sepharose 2B as described in our previous paper (Cöster & Fransson, 1981). The difference between the two preparations is that A was exposed to 8 M-urea for approx. 2 days, whereas B was not. There was no detectable difference in degree of purity.

Total scleral proteoglycan, purified by density-gradient centrifugation, was digested with papain and the side chains (iduronate/glucuronate = 7:13) were isolated by ion-exchange chromatography (Cöster & Fransson, 1981).

Hexuronic acid was determined by the carbazole/borate method (Bitter & Muir, 1962) and protein as described by Lowry *et al.* (1951). Automated versions of these methods were also employed (Heinegård, 1973). Radioactivity was measured in a Packard 2450 liquid-scintillation counter with Insta-Gel as scintillator (0.5 ml of sample and 5 ml of gel).

Reduction and alkylation were performed with dithiothreitol in 4 M-guanidine hydrochloride and with iodoacetamide respectively as described by Cöster & Fransson (1981).

Light-scattering

Details of clarification of solutions, instruments used for measurements of refractive indices and intensities of scattered light, as well as methods of calculations, have been described by Fransson *et al.* (1979). Linear least-squares analyses yielded the extrapolation to $c = 0$ and $\theta = 0$, the values being obtained from the data at 30° , 34° , 37.5° and 45° . Radii of gyration (R_G) were calculated from the limiting slope of the $c = 0$ extrapolate by using the data at 30° , 34° , 37.5° and 45° .

Ultracentrifugation

The experiments were performed with an MSE Centriscan 75 ultracentrifuge. Results were ob-

tained from serial dilutions of stock solutions of proteoglycans in 6 M-guanidine hydrochloride or 0.15 M-NaCl, and each solution was dialysed against the solvent to achieve Donnan equilibrium.

Sedimentation-velocity experiments were conducted at 50000 rev./min at 20°C , by using the schlieren detection system. Sedimentation coefficients were calculated by using the highest point of the schlieren peak as boundary position (Schachman, 1957). Diffusion coefficients were determined by the use of a synthetic-boundary cell at 5000 rev./

Fig. 1. Gel chromatography on Sepharose CL2B of hyaluronate (a), purified proteoglycan (b) and a mixture of hyaluronate and purified proteoglycan (c).

The high-molecular-weight hyaluronate (1 mg) and purified proteoglycan (5 mg) were chromatographed separately (a and b). In the combination experiment (c), 0.1 mg of hyaluronate and 5 mg of proteoglycan were dissolved in 2 ml of 4 M-guanidine hydrochloride, kept at room temperature for 1 h and dialysed against 0.5 M-sodium acetate, pH 7.0, before chromatography. The columns ($9\text{ mm} \times 1300\text{ mm}$) were eluted with the same solvent (acetate) and analysed for protein (—, A_{750} ; Folin method) and uronic acid (----, A_{535} ; carbazole). Abbreviation: V_t , total volume.

min at 20°C. Calculations were made by the 'width-method' as described in the instrument manual (MSE Technical Publication 73). S_{20}^0 and D_{20}^0 were determined by extrapolation to infinite dilution. The best line was fitted by least-squares analyses. Molecular weights were calculated by using the Svedberg equation (Schachman, 1957).

Weight-average molecular weights (M_w) were obtained by meniscus-depletion sedimentation-equilibrium centrifugation (Yphantis, 1964) by using the u.v. detection system. To decrease the time for reaching equilibrium, the centrifuge was initially run at a high speed (50000 rev./min). The apparent molecular weight was calculated from a graph of $\ln c$ versus r^2 , where c is the 'height' of the u.v. tracing and r is the radial distance (Yphantis, 1964).

Dry-weight determinations of proteoglycans were performed by using a Cahn RG electrobalance, with the weighing device inside a high-vacuum system (for details see Axelsson & Heinegård, 1978). Solutions of known proteoglycan content, corrected for moisture, in 6 M-guanidine hydrochloride/5 mM-Tris/HCl, pH 7.0, or in 0.15 M-NaCl/5 M-sodium phosphate, pH 7.4, were used for determinations of partial specific volume by means of a mechanical oscillator (precision densitometer DMA 02C, from Anton Paar K.G., Graz, Austria).

Results

As shown previously (Cöster & Fransson, 1981), proteoglycan I is eluted in a more retarded position

on gel chromatography in 4 M-guanidine hydrochloride than in 0.5 M-sodium acetate, suggesting that proteoglycan I may form aggregates under associative conditions. To ascertain whether multimerization could be mediated by hyaluronate, the purified proteoglycans (according to Method A above) were mixed with hyaluronate in the presence of 4 M-guanidine hydrochloride, dialysed against 0.5 M-sodium acetate and chromatographed on Sepharose CL2B in 0.5 M-sodium acetate (Fig. 1). The presence of high-molecular-weight hyaluronate did not alter the elution position of the two proteoglycans (cf. Figs. 1b and 1c).

As the purified proteoglycan was of rather low buoyant density (Cöster & Fransson, 1981), the possibility that the preparation contained small amounts of endogenous hyaluronate could not be excluded. Therefore attempts to remove such material were undertaken. The purified proteoglycan was mixed with ^3H -labelled hyaluronate under dissociative conditions and subjected to ion-exchange chromatography on a DEAE-cellulose column that was eluted with a linear salt gradient in the presence of 6 M-urea (Fig. 2). An efficient separation of hyaluronate and proteoglycans was obtained. Similar results were obtained when the proteoglycan subfractions I and II were separately chromatographed on the same column. Such purified proteoglycans (I and II) were still unable to interact with hyaluronate in experiments like those in Fig. 1.

It could be argued that a putative hyaluron-

Fig. 2. Ion-exchange chromatography of purified proteoglycan and ^3H -labelled hyaluronate in the presence of 6 M-urea. The purified proteoglycan (30 mg) and ^3H -labelled hyaluronate (10000 d.p.m.) were dissolved in 0.10 M-sodium acetate/6 M-urea, pH 5.8, and chromatographed on a column (8 mm $\times$ 150 mm) of DE-32 DEAE-cellulose that was precycled as recommended by the manufacturer and finally equilibrated with the starting buffer. Elution was performed with a linear gradient (0.10–1.50 M-sodium acetate/6 M-urea, pH 5.8; total volume 100 ml) at a rate of 5 ml/h. The effluent was analysed for uronic acid (—, A_{535} ; carbazole), protein (---, A_{280}) and radioactivity (— · —, ^3H). The shape of the gradient was checked by conductivity measurements (·····).

ate-binding region in the proteoglycan might be irreversibly denatured during chromatography on DEAE-cellulose in the presence of urea (Method A). Therefore scleral proteoglycans were purified by two consecutive dissociative density-gradient centrifugations (Method B above) under conditions that would preserve the aggregatability of cartilage proteoglycan monomers (Hascall & Heinegård, 1974a). The gel-chromatography properties of this preparation of scleral proteoglycans were also not affected by the addition of hyaluronate. In addition, a preparation that had been purified by Method B and subjected to removal of endogenous hyaluronate (as in Fig. 2) did not aggregate in the presence of hyaluronate.

Ultracentrifugation

To obtain a molecular weight for the proteoglycan monomers, ultracentrifugation experiments were performed in 6.0 M-guanidine hydrochloride. In sedimentation-velocity runs there was a single symmetrical peak in both cases, which persisted throughout the experiment. Plots of the logarithm of the radial distance of the boundary position versus time were linear. The sedimentation and diffusion coefficients and the partial specific volumes obtained for the two proteoglycan subfractions are given in Table 1, and the concentration-dependence of the molecular weight is shown in Fig. 3. The values calculated were 160 000–180 000 and 80 000–100 000 for proteoglycans I and II respectively. In sedimentation-equilibrium experiments with proteoglycan I a linear plot of $\ln c$ versus r^2 was obtained, whereas that with proteoglycan II was non-linear. In the former case a molecular weight of 190 000–220 000 was calculated by extrapolation to zero concentration (Fig. 4a). In the latter case the line that gave the highest estimate of molecular weight was used (Fig. 4b), as sedimentation-equilibrium determinations tend to give a minimum weight-average molecular weight. The value calculated for proteoglycan II was 70 000–100 000. Thus the molecular masses of the two proteoglycan

fractions I and II were 160 000–220 000 and 70 000–100 000 daltons respectively. As the protein contents of the two proteoglycans were 45% and 60% respectively, it may be calculated that proteoglycan I contained, on average, four to five dermatan sulphate side chains, whereas proteoglycan II should contain one or two. The weight-average molecular weight of the side chains was 24 000, as determined by light-scattering. In addition, the

Fig. 3. Plot of apparent molecular weight (sedimentation-velocity) versus concentration of (a) proteoglycan I and (b) proteoglycan II in 6 M-guanidine hydrochloride. The molecular weight ($\bar{M}_w$) was calculated for each concentration by the Svedberg equation (see the Experimental section). The rotor speed was 50 000 rev./min. $\bar{v}$ = 0.68 and 0.69 ml/g for proteoglycans I and II respectively. The $\bar{M}_w$ values extrapolated to zero concentration were 170 000 (proteoglycan I) and 89 000 (proteoglycan II) respectively.

Table 1. Ultracentrifugation data for the proteoglycan subfractions in 6 M-guanidine hydrochloride. Sedimentation and diffusion coefficients were obtained as described in the Experimental section (sedimentation-velocity). Molecular weights were calculated from data obtained after sedimentation-velocity (sed. vel.) and sedimentation-equilibrium (sed. eq.) runs. See also Figs. 3 and 4.

Proteoglycan	$10^{-3} \times \bar{M}_w$			$\bar{v}$			
	$10^{13} \times s_{20,w}^0$ (s)	$10^7 \times D_{20,w}^0$ (cm ² /s)	6 M-Guanidine hydrochloride	sed. eq.		(ml/g)	
				sed. vel.	sed. eq.	sed. vel.	sed. eq.
I	2.76	1.25	170 ± 10	6 M-Guanidine hydrochloride	0.15 M- NaCl	0.68	0.66
II	2.21	1.94	89 ± 10	6 M-Guanidine hydrochloride	0.15 M- NaCl	0.69	0.68

molecules also contained an unspecified number of oligosaccharides (Cöster & Fransson, 1981).

Ultracentrifugation experiments (sedimentation-equilibrium) in 0.15M-NaCl also yielded a linear relationship between $\ln c$ and r^2 for proteoglycan I, whereas that for proteoglycan II was non-linear. Extrapolations to zero concentration (Figs. 4c and 4d) gave a molecular weight of 500 000–800 000 for

fraction I and 90 000–110 000 for fraction II. In the latter case, extrapolations from both of the linear portions of the plot of $\ln c$ versus r^2 gave the same apparent molecular-weight range after extrapolation to zero concentration. Centrifugations at higher speeds also produced non-linear curves, but the calculated molecular weights were lower.

Light-scattering

The light-scattering data obtained from solutions of proteoglycans I and II are compiled in Table 2, and representative Zimm plots are shown in Fig. 5. Proteoglycan I exhibited an association phenomenon on gel chromatography in 0.5M-sodium acetate (Cöster & Fransson, 1981). Therefore light-scattering measurements were also performed in this solvent. The particle weights obtained were very high (12×10^6 and 39×10^6 daltons respectively). In 0.15M-NaCl the two proteoglycan subfractions gave particle weights of 3.1×10^6 and 3.4×10^6 daltons respectively. Attempts to dissociate the aggregates were made by using increasing concentrations of guanidine hydrochloride, by increasing the ionic strength and by changing the ionic composition. As shown in Table 2, the particle weight of proteoglycan I was greatly decreased by increasing concentrations of guanidine hydrochloride, especially in the 1–2M interval, and reached a molecular-weight value of 410 000 in 6.0M-guanidine hydrochloride. Aggregates of proteoglycan II began to dissociate already in the 0.5–1.0M-guanidine hydrochloride interval and reached a value of 130 000 in 6.0M-guanidine hydrochloride. Decreasing the pH from 8 to 5 in 0.15M-NaCl did not alter the particle weight. The particle weights of proteoglycans I and II in 0.15M-NaCl were not affected by the addition of hyaluronate or hyaluronate oligo-

Fig. 4. Plot of apparent molecular weight (sedimentation-equilibrium) versus concentration of proteoglycan I (a and c) and proteoglycan II (b and d) in 6M-guanidine hydrochloride (a and b) and in 0.15M-NaCl (c and d). The molecular weight (M_w) was calculated for each concentration (see the Experimental section). For proteoglycan I, equilibrium was attained at 8000 and 5100 rev./min in 6M-guanidine hydrochloride and 0.15M-NaCl respectively. Corresponding values for proteoglycan II were 15 000 and 13 000 rev./min. $\bar{v} = 0.66$ and 0.68 ml/g for proteoglycans I and II respectively in 0.15M-NaCl. The M_w values obtained by extrapolation of zero concentration are given in Table 1.

Table 2. Light-scattering data from the proteoglycan subfractions under various conditions							
The proteoglycan subfractions (I and II) were obtained by gel chromatography. The refractive-index increment ($\Delta n/\Delta c$) values were obtained at 436 nm; R_G is the radius of gyration.							
Conditions (all at pH 7.4 if not stated otherwise)	Proteoglycan ...	I			II		
		$\Delta n/\Delta c$ (ml/g)	R_G (nm)	$10^{-6} \times \bar{M}_w$	$\Delta n/\Delta c$ (ml/g)	R_G (nm)	$10^{-6} \times \bar{M}_w$
0.50M-Sodium acetate, pH 7.0*		0.16	190	12	0.16	437	39
0.15M-NaCl		0.15	106	3.1	0.15	171	3.4
0.15M-KCl		0.15	121	3.2	0.15	161	2.8
1.0M-KCl/0.02M-EDTA		0.15	176	2.9	0.15	243	2.3
0.50M-Guanidine hydrochloride		0.17	225	9.0	0.15	246	11
1.0M-Guanidine hydrochloride		0.16	208	10	0.16	203	2.0
2.0M-Guanidine hydrochloride		0.15	89	1.3	0.16	124	0.65
4.0M-Guanidine hydrochloride		0.14	69	0.42	0.15	97	0.20
6.0M-Guanidine hydrochloride		0.14	69	0.41	0.15	94	0.13
0.15M-NaCl + dermatan sulphate					0.15	314	185

* Mean of four determinations.

Fig. 5. Zimm plots of the light-scattering data from solutions of proteoglycan I in 0.5 M-sodium acetate, pH 7.0 (a), and in 6 M-guanidine hydrochloride/1 mM-sodium phosphate/1 mM- Na_2EDTA , pH 7.4 (b), and from solutions of proteoglycan II in 0.5 M-sodium acetate, pH 7.0 (c), and in 6 M-guanidine hydrochloride/1 mM-sodium phosphate/1 mM- Na_2EDTA , pH 7.4 (d). The following concentrations were used: (a) 0.09, 0.13, 0.8 and 0.22 mg/ml, (b) 0.39, 0.56, 0.73 and 0.90 mg/ml, (c) 0.10, 0.40, 0.60 and 0.80 mg/ml, and (d) 1.10, 2.20, 3.30 and 4.40 mg/ml.

saccharides (decasaccharides or larger). Nor were the properties altered by filtration through Millipore membranes immediately before the centrifugation step in the preparation for light-scattering measurements.

Attempts to remove endogenous hyaluronate from the proteoglycan preparations were made by ion-exchange chromatography (Fig. 2). The purified subfractions yielded particle weights in 0.15 M-NaCl (Table 3) similar to those before the ion-exchange step (Table 2). Attempts to destroy a putative hyaluronate-binding site were made by reduction and alkylation. It was previously observed that reduction and alkylation abolished aggregation as seen in gel chromatography (Cöster & Fransson, 1981). However, reduced and alkylated proteoglycans displayed pronounced aggregation when studied by light-scattering (Table 3). For proteoglycan II the particle weight increased by one order of magnitude after reduction and alkylation.

As free dermatan sulphate chains display self-association that can be affected by ionic strength and ionic composition (Fransson *et al.*, 1979), we attempted to dissociate the scleral proteoglycans in this way. As shown in Table 2, changing the cation from Na^+ to K^+ had little or no effect on the particle weight. Increasing the ionic strength from 0.15 M-KCl to 1.00 M-KCl and including EDTA changed the

Table 3. Light-scattering data from the proteoglycan subfractions after ion-exchange chromatography or reduction and alkylation

The proteoglycan subfractions (I and II) were obtained by gel chromatography. The fractions were separately chromatographed on DEAE-cellulose (DE32) as in Fig. 2 or reduced and alkylated (RA). All measurements were performed in 0.15 M-NaCl/1 mM-sodium phosphate/1 mM- Na_2EDTA , pH 7.4.

Sample	$\Delta n/\Delta c$ (ml/mg)	R_G (nm)	$10^{-6} \times \bar{M}_w$
I-DE32	0.15	95	3.0
II-DE32	0.15	177	2.8
I-RA	0.15	145	3.7
II-RA	0.15	295	27

particle weights from 3.2×10^6 to 2.9×10^6 daltons for proteoglycan I and from 2.8×10^6 to 2.3×10^6 daltons for proteoglycan II. When considering the effect of ionic strength, it should be noted that in 0.5 M-guanidine hydrochloride both proteoglycans yielded particle weights that were higher (9×10^6 and 1.1×10^7 daltons respectively) than those obtained at physiological ionic strength.

The dermatan sulphate side chains of total scleral proteoglycan were also studied by light-scattering. The chains gave a particle weight of 100 000 daltons

in 0.15 M-NaCl. In 0.15 M-KCl scleral dermatan sulphate chains were only partially dissociated ($M_w = 75\,000$). In the presence of 1.00 M-KCl/0.02 M-EDTA the preparation yielded a 'monomeric' molecular-weight value (24 000). To test whether multimerization of the proteoglycans was generated by interaction between the dermatan sulphate side chains, attempts to compete for binding were made by addition of free chains (10-fold molar excess) to solutions of proteoglycan II in 0.15 M-NaCl. Surprisingly, the addition of free chains produced a great increase in the intensity of scattered light. After subtraction of the intensities derived from the free chains, it was calculated that the proteoglycans had increased their particle size approx. 50–60-fold.

Discussion

As shown by gel chromatography (Fig. 1), the proteoglycan fractions were not physically homogeneous; in particular, proteoglycan I contained species of very different molecular sizes. This is illustrated by the range of molecular weights (160 000–410 000) obtained for this material in 6 M-guanidine hydrochloride by using the s/D approach, sedimentation velocity or light-scattering. The s/D method yielded the lowest and light-scattering the highest estimates. This is in accordance with theoretical considerations as s/D yields a molecular-weight value somewhere between number- and weight-average under the present circumstances, whereas light-scattering is especially sensitive to the presence of large species. Therefore proteoglycan I may well include species with more than five side chains per protein core.

The smaller proteoglycan (II) appeared more homogeneous on gel chromatography (Fig. 1) than the larger one, and the molecular-weight values obtained ranged between 70 000 and 130 000. As the size of the core protein was 46 000 daltons, a maximum of three side chains can be accommodated (Cöster & Fransson, 1981).

The present results also confirm and extend previous indications of a self-association among proteodermatan sulphate molecules (Cöster & Fransson, 1981). The techniques used to study self-association in previous and the present investigations have been gel chromatography, ultracentrifugation and light-scattering. Before discussing the results it is useful to consider the parameters to which the various techniques are sensitive. Gel chromatography, by virtue of the dilution of the sample as the elution proceeds and the involvement of a certain rate of shear depending on the flow rate, would be expected to be sensitive only to strong associations. In ultracentrifugation experiments the concentration of the proteoglycan can be con-

trolled, and therefore even weak associations should be detected, but transport phenomena may still affect the result. Light-scattering, finally, is the only technique where the proteoglycan solution is not perturbed by shear and/or pressure. However, the light-scattering technique is especially sensitive to the presence of large particles. Even if only a small proportion of the total material participates in the formation of a large aggregate, the weight-average molecular weight of the preparation will be greatly increased.

Proteodermatan sulphate I showed a small but significant tendency to aggregate on gel chromatography under associative conditions. The aggregation was not promoted by the presence of hyaluronate. The smaller proteoglycan (II) had the same elution position in associative as well as in dissociative solvents. In the ultracentrifuge, proteodermatan sulphate I exhibited a 3–4-fold increase in size under associative conditions, whereas proteodermatan sulphate II was only marginally affected. The latter proteoglycan displayed association only in light-scattering experiments. In 0.15 M-NaCl the two proteodermatan sulphates gave particle weights in excess of 3×10^6 daltons (see also Öbrink, 1972). The formation of these super-aggregates was not promoted by the presence of hyaluronate, nor were the aggregates sensitive to a decrease in pH from 8 to 5 (Hardingham & Muir, 1972, 1973, 1974). The particle weights obtained for the proteodermatan sulphates in light-scattering under associative conditions greatly exceeded the values estimated from gel-chromatography experiments. This might suggest that only a limited number of molecules participated in the formation of a very large aggregate. It is also possible that the discrepancy between the light-scattering and the gel-chromatography data is due to the presence of super-aggregates that are sensitive to shear, in analogy with previous findings with free chains (Fransson *et al.*, 1979). It was also noted that, in ultracentrifugation experiments, the apparent molecular weight decreased with concentration. As the centrifuge was 'over-speeded' before equilibrium was attained, the possibility that the sample was depleted of high-molecular-weight aggregates cannot be excluded. Further systematic ultracentrifugal studies might provide results that shed light on this problem.

Free dermatan sulphate chains self-associate in the presence of 0.15 M-NaCl (Fransson, 1976; Fransson & Cöster, 1979; Fransson *et al.*, 1979). The dermatan sulphate side chains of scleral proteoglycans also associate under the same conditions. Therefore an interaction between the side chains of proteodermatan sulphates might enable the molecules to multimerize. The scleral proteoglycans and their dermatan sulphate side chains did not dissociate under identical conditions. These results

could be interpreted to indicate that multimerization was not solely an effect of chain-chain interaction. However, as multimerization would involve the interaction of several side chains, a co-operative effect might render the proteoglycan more stable to dissociating solvents than are the corresponding chains. Addition of a large excess of free dermatan sulphate chains to scleral proteoglycan increased the particle size of the proteoglycan 50–60-fold. Free chains may have interacted with several proteoglycan monomers, bridging between them to form a large network. This interpretation requires the presence of two or more binding sites on each glycan chain, which is a distinct possibility (Fransson & Cöster, 1979).

The scleral proteodermatan sulphates have a small number of side chains, and hence the protein core may not be constrained to an extended conformation. Therefore the conformation of the protein core of proteodermatan sulphate may affect the aggregatability, e.g. by controlling the spatial arrangement of the dermatan sulphate side chains. Reduction and alkylation of the scleral proteoglycan I resulted in loss of aggregatability in gel-chromatography experiments, whereas the aggregatability was unchanged when studied by light-scattering. Reduced and alkylated proteoglycan II formed aggregates that were approx. 10 times larger than were those formed by the untreated material. We do not have any plausible explanations of these phenomena.

In conclusion, sclera contains two proteodermatan sulphates, one larger, which exhibits self-association both in gel-chromatography and light-scattering experiments, and one smaller, which showed aggregation only in light-scattering experiments. We propose that the aggregation is solely or partly mediated by interaction between the dermatan sulphate side chains. However, our data do not exclude other forms of self-association, e.g. via polysaccharide-protein and protein-protein interactions.

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