Isopycnic-centrifugation studies in caesium chloride and in caesium sulphate on dermatan sulphate proteoglycans from bovine sclera

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1. Two proteodermatan sulphate species from bovine sclera (fractions PG-I and PG-II) separable by gel chromatography were studied by isopycnic centrifugations in CsCl and Cs₂SO₄ both in an analytical and a preparative mode. 2. In CsCl, fraction PG-I formed a broad band at a density $\rho = 1.75$ g/ml whereas fraction PG-II banded sharply at $\rho = 1.64$ g/ml. However, in Cs₂SO₄, fraction PG-II banded at $\rho = 1.51$ g/ml and fraction PG-I at $\rho = 1.40$ g/ml, a reversal of the relative banding positions of the two species in CsCl. 3. Preparative isopycnic centrifugations in the two caesium salts permitted further subfractionation of fractions PG-I and PG-II. In both CsCl and Cs₂SO₄ fraction PG-I was split into subfractions that varied greatly in uronate/protein ratios but had very similar uronate composition. In contrast, isopycnic centrifugation of fraction PG-II in Cs₂SO₄ gave rise to subfractions with similar uronate/protein ratios but markedly different uronate composition (iduronate content, 88–42%). 4. Subfractions of fractions PG-I and PG-II obtained in preparative centrifugations in CsCl or Cs₂SO₄ were examined in the analytical ultracentrifuge. These subfractions banded at discrete positions in the gradient. Estimations of apparent molecular weight for these subfractions from data from the analytical isopycnic centrifugations gave values that were much higher (around 1×10^6) than were those obtained previously for their 'monomeric' states (fraction PG-I 160 000–410 000, and fraction PG-II 70 000–130 000). 5. In CsCl, fraction PG-I may be subfractionated according to the number of side chains in the molecule. Fraction PG-I increased its net solvation (i.e. lowered its buoyant density) to a larger extent than did fraction PG-II in going from CsCl to Cs₂SO₄ (i.e. from lower to higher water activity). It is proposed that the presence of large amounts of iduronate in fraction PG-II makes these molecules relatively less solvated in Cs₂SO₄. Thus the uronate composition may be an important factor in determining the banding position of proteodermatan sulphates in density-gradient centrifugations.

Two populations of proteodermatan sulphate from bovine sclera have been chemically and physically characterized (Cöster & Fransson, 1981; Cöster *et al.*, 1981). The smaller one had an apparent molecular weight of 70 000–130 000 with a protein core of 47 000, whereas the larger one had a molecular weight of 160 000–410 000. The proteoglycans were polydisperse, presumably owing to variable substitution of the protein cores with dermatan sulphate and oligosaccharides. The dermatan sulphate chains of the smaller proteoglycan were more iduronate-rich than were those of the larger one.

In the present work attempts to subfractionate these proteoglycans further were made by using isopycnic centrifugations in caesium salts. This technique was introduced in the proteoglycan field by Sajdera & Hascall (1969) for purifying cartilage proteoglycans. However, most of the cartilage proteoglycans have too high a buoyant density to band in CsCl. Numerous studies on nucleic acids have shown that the buoyant density of those macromolecules varies with the gradient-forming medium; for a review see Vinograd (1963). An increase in water activity is accompanied by a decrease in the buoyant density of DNA. Mashburn

et al. (1974) showed that cartilage proteoglycans could be banded in Cs_2SO_4 , and Bonnet *et al.* (1980) used this property to study the polydispersity and heterogeneity within cartilage proteoglycans. The introduction of a third component such as guanidinium chloride in addition to the caesium salt has complex effects on the anhydrous density of the macromolecule and on the selective solvation. Bonnet *et al.* (1980) noted an increased density of the proteoglycans when guanidinium chloride was used together with Cs_2SO_4 . On the other hand Mashburn *et al.* (1974) showed that high concentrations of guanidinium ion in CsCl gradients make cartilage proteoglycans more buoyant. In the present study dermatan sulphate proteoglycans were centrifuged isopycnicly in CsCl or Cs_2SO_4 alone.

Experimental

Materials

Stock solutions of guanidinium chloride (8M; technical grade; Sigma) were treated with active charcoal to decrease the $A_{280}^{1\text{cm}}$ to less than 0.15. Solutions of CsCl and Cs_2SO_4 were treated in a similar way to remove a slight haze from the solutions. Stock solutions of urea (8M) were passed through a mixed ion-exchanger to remove traces of cyanate. Buffers containing 6M-urea made up from the purified stock were kept at 4°C and used within 5 days. Chondroitinase ABC (EC 3.2.1.35) was obtained from Miles Laboratories. Sepharose CL-2B was bought from Pharmacia and DE-52 DEAE-cellulose from Whatman.

Analytical methods

Densities were measured with a 200 μl pipette as pycnometer. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Uronic acid content was determined by the carbazole method as described by Dische (1947) and by the orcinol method with a 20 min boiling time (Brown, 1946). In both cases D-glucuronolactone was used as a standard. In some cases an automated carbazole method was used (Heinegård, 1973). The proportions of L-iduronate and D-glucuronate were estimated from carbazole/orcinol ratios (0.2:1.0 for 100% L-iduronate and 2.2:1.0 for 100% D-glucuronate of total uronate) and by determining the amount of unsaturated glycuronosyl residues formed after digestions with chondroitinase AC and chondroitinase ABC (Cöster & Fransson, 1981). Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of the protein cores was performed as described by Cöster & Fransson (1981). Gel chromatography of the core proteins was conducted on a column (6 mm $\times$ 1200 mm) of Sepharose CL-6B. The column was eluted with 4M-guanidinium

chloride/50 mM-sodium acetate buffer, pH 5.8, at a rate of 2 ml/h. The effluent was monitored by an Uvicord S detector (LKB-2138) set at 280 nm, and 700 μl fractions were collected with the aid of a drop-counter.

Purification of proteoglycans

Proteoglycans were extracted from bovine sclera with 4M-guanidinium chloride/50 mM-sodium acetate, pH 5.8/0.10M-6-aminohexanoic acid/10 mM-disodium EDTA/5 mM-benzamidine chloride/5 mM-N-ethylmaleimide as described by Cöster & Fransson (1981). After removal of non-solubilized material by centrifugation, CsCl was added to the supernatant until a density of 1.35 g/ml was reached. The samples were centrifuged in an MSE 65 centrifuge (8 $\times$ 25 ml angle rotor; 36000 rev./min; 60 h; 12°C). Tubes were pierced and emptied into ten fractions, which were analysed for uronate content and density. Fractions from two runs with a density greater than 1.33 g/ml were pooled (90–95% of the total uronate) and subjected to a second density-gradient centrifugation under the same conditions (Fig. 1). The bottom two fractions, which had a density equal to or greater than 1.43 g/ml and contained 60% of the total uronate, were pooled. This material was dialysed against 0.2M-acetate/6M-urea, pH 5.8, and subjected to ion-exchange chromatography on a column (20 mm $\times$ 360 mm) of DE-52 DEAE-cellulose. The column was eluted with a gradient of acetate/6M-urea, pH 5.8, with a flow rate of 30 ml/h, and 10 ml fractions were collected. The mixing vessel contained 1 litre of 0.2M-acetate/6M-urea, pH 5.8, and the reservoir contained 1 litre of 1.5M-acetate/6M-urea, pH 5.8. The effluent was analysed by an automated version of the carbazole reaction and by the absorbancies at 260 and 280 nm (results not shown). The uronate-containing fractions were pooled, concentrated by ultrafiltration on a PM-30 filter in an Amicon ultrafiltration cell and then dialysed against 4M-guanidinium chloride/50 mM-acetate buffer, pH 5.8. The proteoglycans were subsequently chromatographed on Sepharose CL-2B in 4M-guanidinium chloride/50 mM-acetate buffer, pH 5.8, which separated them into two major fractions. These were pooled as indicated in Fig. 2(a), concentrated and re-chromatographed individually on the same column (Figs. 2b and 2c). The major portions of the respective proteoglycans were pooled as indicated. The smaller proteoglycans of the larger population (fraction PG-I) were combined with the larger ones of the smaller population (fraction PG-II). This material was designated fraction PG-M. The three proteoglycan fractions (PG-I, PG-II and PG-M) were concentrated and stored frozen in 4M-guanidinium chloride/50 mM-acetate buffer, pH 5.8.

Fig. 1. Second CsCl-density-gradient centrifugation in 4M-guanidinium chloride of proteoglycans extracted from bovine sclera

Proteoglycans were extracted from bovine sclera and subjected to a first CsCl-density-gradient centrifugation as described in the Experimental section. Fractions with a density greater than 1.33g/ml were pooled and subjected to a second density-gradient centrifugation under the same conditions. The fractions were monitored for uronate (—) with the carbazole reaction, for protein (----) by A_{280} and for density (O). The bottom two fractions, which contained 60% of the total uronate, were pooled. For experimental details see the text.

Analytical ultracentrifugation

The proteoglycan fractions were subjected to analytical isopycnic density-gradient ultracentrifugation in an MSE Centriscan 75 with a six-place aluminium rotor and 1 cm epoxy cells fitted with quartz windows. All experiments were performed at 25°C. Samples of proteoglycans were dialysed first against water and then against the appropriate caesium salt. The initial density was determined by pycnometry. Solutions of fractions PG-I and PG-II had relative A_{280} against the diffusate of 0.1, equivalent to approx. 0.3 mg of proteoglycan/ml. The PG-M fraction was adjusted to a starting A_{280} of 0.2, corresponding to approx. 0.5 mg/ml. Samples (380 μ l) were centrifuged against 410 μ l of the appropriate blank (sample column length approx. 1 cm). Equilibrium runs were typically conducted for

Fig. 2. Gel chromatography on Sepharose CL-2B of proteoglycans obtained after ion-exchange chromatography

Proteoglycans were prepared as described in the Experimental section. After ion-exchange chromatography the proteoglycans were pooled and dialysed against 4M-guanidinium chloride/50 mM-acetate buffer, pH 5.8, and applied to the column (a). The effluent was analysed for uronate by the orcinol method (A_{670} , —) and for nucleic acids by A_{260} (----). Material was pooled as indicated by horizontal bars. The two proteoglycan fractions were rechromatographed on the same column: the larger component in (b) and the smaller component in (c). The proteoglycans in (b) and (c) were pooled in one major portion and one minor portion, as indicated by the vertical lines. The minor portions in (b) and (c) were pooled together and designated fraction PG-M. The size of the column was 40 mm $\times$ 1470 mm and it was operated with 4M-guanidinium chloride/50 mM-acetate buffer, pH 5.8, at a rate of 25 ml/h. Fractions of volume 22 ml were collected and analysed for uronate by the orcinol reaction. For experimental details see the text.

24 h, but scans at 16 h and 18 h were used to follow the progress towards equilibrium.

Preparative ultracentrifugation of subfractions

Preparative equilibrium ultracentrifugation was performed in the MSE 65 ultracentrifuge fitted with the 8×25 ml rotor. Proteoglycan fractions were dialysed against water and adjusted to the appropriate starting density by the addition of solid CsCl or Cs_2SO_4 . Sealed tubes containing 16.5 ml of solution at starting concentration of approx. 0.6 mg/ml were used for each experiment, which was run for 60 h at 36 000 rev./min and 15°C . Each centrifuge tube was clamped in a stand and a cap with a central hole was placed over the top of the tube. A fine glass capillary was carefully lowered through the cap to the bottom of the tube, and the gradient was pumped through the detector (LKB Uvicord S set at 280 nm) by using an LKB Varioperpex II peristaltic pump, and 250 μl fractions were collected by using a drop-counter. As the gradient was emptied from the bottom of the tubes, the solution was passed through the Uvicord from top to bottom to avoid gradient inversion. Thus the detector and the capillary tubing had to be pre-filled with a solution that was denser than the highest density in the gradient. The fractions were analysed for density and uronate.

Calculations of buoyant density and apparent molecular weight

The relationship between the density gradient, $d\rho/dr$, the radial distance, r , and the rotor speed, ω , for a particular caesium salt is expressed by the equation:

$$\beta = \frac{\omega^2 r}{d\rho/dr} \quad (1)$$

where β is the salt-distribution parameter as defined by Ifft *et al.* (1961). The buoyant density at a particular radial distance from the rotor centre (ρ_r) was calculated from the equation:

$$\rho_r = \rho_e + (d\rho/dr)_{r_e} \cdot (r - r_e) \quad (2)$$

where ρ_e is the starting density. The apparent iso-concentration point, r_e , is given by the expression:

$$r_e = \frac{r_b^2 + r_m^2}{2} \quad (3)$$

where r_b and r_m are the radial distances from the rotor centre to the bottom of the tube and to the meniscus of the liquid column respectively. The liquid column was considered short enough to assume a constant value for β over the gradient, and values from Vinograd & Hearst (1962) and from Ludlum & Warner (1965) were used for CsCl and Cs_2SO_4 respectively. The distribution of molecules in

the gradient is related to the molecular weight by the equation:

$$M = \frac{RT\rho_s}{\sigma^2\omega^2 r_s \left(\frac{d\rho}{dr} \right)_r} \quad (4)$$

where ρ_s is the buoyant density obtained from eqn. (2), R the universal gas constant, T the absolute temperature, r_s the radial position and σ the standard deviation of the band. The value for the latter was estimated as the half-width of the peak at 0.607 times the height. Polydispersity, heterogeneity and non-ideality effects make the distribution broader than what is determined by the size of the macromolecules alone. This effect gives rise to low estimates of the apparent molecular weight. Accurate calculations requires knowledge of the 'effective' density gradient, which takes into account solvation and compression effects on the macromolecules. In the present work eqn. (4) was applied with this correction omitted. For a more extensive treatment, see, e.g., Creeth & Denborough (1970).

Results

The centrifugation step (Fig. 1) in the purification procedure for proteoglycans resulted in removal of most of the extraneous proteins. The ion-exchange chromatography step (results not shown) separated the rest of the proteins and most of the nucleic acids from the proteoglycans, as judged from A_{260} , A_{280} and carbazole analyses. Finally, a separation of the two proteoglycan populations and an almost complete removal of nucleic acids from the proteoglycans was afforded by chromatography on Sepharose CL-2B (Fig. 2a). Re-chromatography on the same gel yielded the fractions PG-I, PG-II and PG-M, the last being a mixture of small molecules from fraction PG-I and larger ones from fraction PG-II (Figs. 2b and 2c). Disc gel electrophoresis and gel chromatography after chondroitinase-ABC digestions showed that the smaller PG-II proteoglycan contained the small protein core with a molecular weight of 47 000. The larger PG-I proteoglycan contained the larger protein core, the molecular weight of which could not be assessed by gel electrophoresis as it did not enter the gel. No cross-contamination between fractions PG-I and PG-II was observed.

Analytical isopycnic-centrifugation patterns of the proteoglycans are shown in Fig. 3. In CsCl (Fig. 3a) fraction PG-II banded sharply at a density $\rho = 1.64$ g/ml, whereas fraction PG-I formed a broad band at $\rho = 1.75$ g/ml. Fraction PG-M had a heavy component of the PG-II type, but its PG-I component was so broad that it was barely

detectable above the base-line. However, in Cs_2SO_4 (Fig. 3b) fraction PG-II banded at a density of $\rho = 1.51 \text{ g/ml}$ and fraction PG-I at $\rho = 1.40 \text{ g/ml}$. Thus there was a reversal of the relative positions of fractions PG-I and PG-II in the density gradient when going from CsCl to Cs_2SO_4 . In the latter case (Fig. 3b) the shape of the profiles for both fractions PG-I and PG-II indicated that more than one component was present. In fraction PG-I most of the material was in the lighter component, whereas in fraction PG-II the heavier one formed the major fraction. Fraction PG-M contained components corresponding to the major ones of fractions PG-I and PG-II. These components were lighter and heavier respectively than the main components of fractions PG-I and PG-II.

Fig. 3. Analytical density-gradient centrifugation of fractions PG-I (—), PG-II (---) and PG-M (.....)

(a) Centrifugation was performed in CsCl at a starting density of 1.65 g/ml and a rotor speed of 57000 rev./min (Centriscan 75) at 25°C . The meniscus and the bottom of the tube are marked with (↓) and the position where DNA would band is marked with (▼). (b) Centrifugation was performed in Cs_2SO_4 at a starting density of 1.43 g/ml and a rotor speed of 50000 rev./min (Centriscan 75) at 25°C . For experimental details see the text.

Fig. 4. Continuous monitoring at A_{280} of a preparative density-gradient centrifugation of scleral proteoglycans. The fraction PG-M was centrifuged in Cs_2SO_4 at a starting density of 1.45 g/ml for 60 h at 36000 rev./min (MSE 65) and 15°C . The tube was emptied through the LKB Uvicord S detector set at an absorbance scale of $0-2$. The fraction collector was connected to the chart recorder, and the fraction changes are noted by a mark on the trace. The results of carbazole analysis (A_{535} ; ----) are shown above the A_{280} (—) tracing in arbitrary units. For experimental details see the text.

Results from preparative experiments are shown in Figs. 4 and 5. The fractions obtained were analysed for uronate by the carbazole method, and a close correspondence to the A_{280} was found. Fig. 5 shows the results obtained from preparative centrifugations of fractions PG-I and PG-II in CsCl and Cs_2SO_4 respectively. Fractions were pooled as indicated in the legend to Fig. 5, and analytical data are shown in Table 1. In CsCl fraction PG-I was split into three subfractions (Fig. 5a) with uronate (carbazole)/protein ratios of $1.35:1.0$, $0.98:1.0$ and $0.65:1.0$ respectively. The iduronate/glucuronate ratios of these subfractions were similar, i.e. $16-21\%$ L-iduronate of total uronate. Fraction PG-II was split (Fig. 5a) into one sharp major (94%) and one broad minor (6%) subfraction with greatly different carbohydrate/protein ratios. The major subfraction had a high L-iduronate content (76%). In Cs_2SO_4 (Fig. 5b) fraction PG-I was split into five subfractions with uronate (carbazole)/protein ratios ranging from $1.37:1.0$ to $0.61:1.0$. Again, the uronate composition of the major subfractions was similar, i.e. $10-20\%$ L-iduronate of total uronate. Isopycnic centrifugation of fraction PG-II in Cs_2SO_4 (Fig. 5b) gave rise to five subfractions, all of which had very similar uronate/protein ratios [$0.20-0.22:1.0$ as uronate (carbazole)/protein and $0.32-0.47:1.0$ as uronate (orcinol)/protein]. However, these subfractions had greatly different uronate compo-

Fig. 5. Preparative equilibrium centrifugation of fractions PG-I (—) and PG-II (----).

(a) Centrifugation was performed in CsCl at a starting density of 1.68 g/ml and a rotor speed of 36000 rev./min (MSE 65) for 60 h at 15°C. The analyses were orcinol reaction for uronate (A_{670}) and density (●—●). The PG-II fraction (----) banded sharply as an opalescent band clearly visible in the centrifuge tube. It was not possible to recover the material quantitatively, as it adhered to the walls of the centrifuge tube. With a normal recovery the peak should be much higher (.....), which illustrates the difference in band-width of fraction PG-II compared with fraction PG-I. The PG-I band (—) was pooled into three subfractions: 5–6, 10–25 and 26–40. The material of the PG-II band (----) was pooled into subfractions 5–29 and 30–48. (b) Centrifugation was performed in Cs_2SO_4 at a starting density of 1.44 g/ml and a rotor speed of 36000 rev./min (MSE 65) for 60 h at 15°C. The analyses were carbazole reaction for uronate (A_{535}) and density (●—●). The material of the PG-I band (—) was pooled into five subfractions: 24–30, 31–32, 33–34, 35–37 and 38–44. The material of the PG-II band (----) was also pooled into five subfractions: 16–18, 19–20, 21–23, 24–32 and 33–39. For experimental details see the text.

sitions, as indicated both by the carbazole/orcinol ratios and by estimations of unsaturated glycuronosyl residues liberated by chondroitinase AC (glucuronate) or chondroitinase ABC (glucuronate plus iduronate). The iduronate contents of the

Fig. 6. Heterogeneity of PG-I subfractions in a Cs_2SO_4 density gradient in the analytical ultracentrifuge.

Subfractions 28 (---), 32 (.....), 34 (—) and 36 (----) from the preparative experiment with fraction PG-I in Cs_2SO_4 (Fig. 5b; —) were dialysed against Cs_2SO_4 with a density of 1.38 g/ml. The samples were run simultaneously for 36 h at 50000 rev./min (Centriscan 75). The apparent molecular weights of subfractions 28, 32 and 34 were calculated from the data by using eqn. (4) in the Experimental section. Values of 350000 for subfraction 28, 690000 for subfraction 32 and 789000 for subfraction 34 were obtained. No attempts were made to calculate a molecular weight for subfraction 36, as the shape of the peak was too asymmetric (----). However, the band-width suggests that subfractions 28 and 36 should yield comparable apparent molecular weights. For experimental details see the text.

subfractions derived from the sharp band in Fig. 5(b) were rather similar (approx. 80%), whereas those of subfractions 24–32 and 33–39 were much lower (approx. 63% and 45% respectively). It should be added that all of the PG-II subfractions contained the 47000-mol.wt. core protein.

Heterogeneity within fractions PG-I and PG-II was studied by re-running subfractions of fraction PG-I taken from the preparative gradient in Cs_2SO_4 (Fig. 5b) in the analytical ultracentrifuge (Fig. 6). The results show that species of different buoyant density existed within the bands. Estimates of apparent molecular weights on the basis of eqn. (4) for fractions 28, 32 and 34 obtained after re-centrifugation (Fig. 6) yielded the values 350000, 690000 and 789000 respectively. Re-centrifugations in the analytical ultracentrifuge of subfractions from fraction PG-II (Fig. 5b) displayed a similar heterogeneity (results not shown). Estimations of the apparent molecular weight for the peak fractions 19–20 in Fig. 5(b) gave a value of 1.2×10^6 .

Table 1. *Analyses of proteoglycan fractions obtained after isopycnic centrifugation in CsCl and Cs₂SO₄*

The purified proteoglycans PG-I and PG-II were prepared as described in the Experimental section and then subjected to isopycnic centrifugation in CsCl or Cs₂SO₄ as shown in Fig. 5. Fractions were pooled, dialysed, freeze-dried and analysed. The iduronate content was obtained from enzymic determinations with chondroitinases (see the Experimental section).

Subfraction	Distribution (%)	Protein (%)	Uronate (carbazole reaction) (%)	Carbazole	L-Iduronate
				orcinol ratio	total hexuronate (%)
CsCl gradient					
PG-I 5–6	12	15.4	20.8	1.54	21
PG-I 10–25	72	19.9	19.5	1.47	16
PG-I 26–40	16	25.5	16.7	1.33	17
PG-II 5–29	6	12.1	12.3	0.62	16
PG-II 30–48	94	37.6	8.2	0.42	76
Cs ₂ SO ₄ gradient					
PG-I 24–30	27	10.7	14.7	1.35	14
PG-I 31–32	19	16.4	15.6	1.87	10
PG-I 33–34	32	15.6	12.6	1.27	20
PG-I 35–37	13	16.6	13.0	1.31	15
PG-I 38–44	9	17.9	11.0	1.28	14
PG-II 16–18	9	21.0	4.5	0.46	62
PG-II 19–20	30	32.1	6.6	0.43	79
PG-II 21–23	17	30.1	6.1	0.45	77
PG-II 24–32	27	26.4	5.9	1.01	65
PG-II 33–39	17	13.7	3.0	1.24	42

Discussion

As shown previously (Cöster & Fransson, 1981), sclera contains two types of proteoglycans, one larger (PG-I) and one smaller (PG-II). The smaller one has a higher protein/uronate ratio and a lower carbazole/orcinol ratio (i.e. higher iduronate content) than does the larger. The preparations of fractions PG-I and PG-II used in the present investigation are not exactly comparable with those studied previously (Cöster & Fransson, 1981). In the previous case proteoglycans with density >1.30 g/ml were used (90% of total uronate), whereas in the present scheme material with density >1.43 g/ml (60% of total uronate) was pooled, thus omitting the lighter proteoglycans (mainly PG-II type: A. Malmström, unpublished work). Accordingly, the protein content of this preparation was lower than that of the previous one (Table 1). Furthermore, the use of *N*-ethylmaleimide during preparation served the dual purpose of preventing disulphide exchange and of inhibiting proteinase activities. Nevertheless, the basic differences between fractions PG-I and PG-II are preserved, i.e. the core proteins and the uronate compositions of the dermatan sulphate side chains are different.

The two proteodermatan sulphate populations isolated from sclera differ with regard to the number and type of side-chain substitutions. We have further studied their polydispersity by isopycnic density-gradient centrifugations in CsCl and

Cs₂SO₄. The buoyant density of a proteoglycan in a caesium salt solution is determined by (a) the anhydrous density, which is dependent on the chemical composition, e.g. the protein/carbohydrate ratio, (b) cation–polyanion interaction and (c) 'macromolecular solvation'. The last may be viewed as associations between the proteoglycan and water molecules that lower the density from that of the anhydrous state to the buoyant density actually observed. The extent of solvation is determined by the nature of the caesium salt [generally lower in acetate and higher in sulphate (Vinograd, 1963)] but also by the chemical composition of the macromolecule. The various structural entities interact differently with the solvent molecules.

In the present study fraction PG-II was more buoyant than fraction PG-I in CsCl (Figs. 3a and 5a). This may be ascribed to the relatively high protein/uronate ratio in the former material. Furthermore, fraction PG-I displayed marked polydispersity with regard to buoyant density. As this pool of proteoglycans comprises species with four to ten side chains (Cöster *et al.*, 1981), the anhydrous density of the species may vary as a consequence of differences in the protein/carbohydrate ratio.

One would expect the scleral proteoglycans to have much lower buoyant densities in Cs₂SO₄ than in CsCl, and fraction PG-I moved from an average density of 1.75 g/ml in CsCl to 1.40 g/ml in Cs₂SO₄ (Figs. 5a and 5b), which is similar behaviour to that observed for cartilage proteoglycans [from $\rho > 1.9$ to

$\rho \approx 1.55$ (Bonnet *et al.*, 1980)]. However, fraction PG-II changed its average density from 1.63 g/ml in CsCl to 1.51 g/ml in Cs_2SO_4 , and this smaller change resulted in the reversal of the relative banding positions of fractions PG-I and PG-II in the Cs_2SO_4 gradient compared with those in the CsCl gradient (Figs. 3 and 5). As fraction PG-II did not increase its net solvation to the same extent as did fraction PG-I, in going from CsCl to Cs_2SO_4 there may be opposing contributions from, for example, the carbohydrate portion. Preparative subfractionation of fraction PG-II in Cs_2SO_4 (Fig. 5b) indicated that the variation in side-chain composition, i.e. iduronate/glucuronate ratio, becomes the major determining factor for the buoyant density of fraction PG-II in Cs_2SO_4 (Table 1). The variability observed in fraction PG-II is correlated with uronate composition, whereas the molecules of fraction PG-I vary with regard to the number of side-chain substitutions. Thus, in addition to the protein/carbohydrate ratio, the uronate character may be an important factor in determining the banding position of a proteodermatan sulphate in density-gradient centrifugations.

As mentioned in the Experimental section, the distribution of molecules in a gradient is related to the molecular weight according to eqn. (4). Estimations of apparent molecular weight for hydrated molecules from band-widths is lower than the true molecular weight, owing to polydispersity. In the present work, however, the sharpness of the PG-II band in CsCl (Figs. 3a and 5a) was not consistent with the molecular weight for this material (approx. 100000). When eqn. (4) was applied to the data in Fig. 3(a), an apparent molecular weight of 627000 was obtained for fraction PG-II. We conclude that an aggregation of molecules of fraction PG-II takes place under these conditions. This is further illustrated in the preparative experiment (Fig. 5a), where a sharp opalescent band was formed. The widths of the major bands of fractions PG-I and PG-II in Cs_2SO_4 were similar, unlike the situation in CsCl (Figs. 3 and 5). The increased sharpness of the PG-I band in Cs_2SO_4 is consistent with the increasing steepness of the gradient due to the lower β -value in Cs_2SO_4 , i.e. 0.65×10^9 compared with 1.19×10^9 for CsCl [see eqn. (1)]. The width of the major PG-II band was little affected by going from CsCl to Cs_2SO_4 . As we may also ascribe increased sharpness of a band to aggregate formation, the results obtained with fraction PG-II suggest that there is a decreased tendency to aggregate in Cs_2SO_4 . The variants that remain aggregated were more iduronate-rich than those that yield a broader distribution. This finding reinforces the notion that solvation of a proteoglycan is decreased by a high iduronate content. By employing eqn. (4) and data such as those in Fig. 6, particle weights of 800000

and 1.2×10^6 were calculated for aggregates of fractions PG-I and PG-II respectively. These values are clearly inconsistent with 'monomer' molecular-weight estimates, i.e. 160000–410000 and 70000–130000 for fractions PG-I and PG-II respectively (Cöster *et al.*, 1981), but are consistent with values obtained under associative conditions by using sedimentation-equilibrium or light-scattering measurements.

In isolating connective-tissue proteoglycans, the major non-protein contaminant is nucleic acid. In the present procedure nucleic acids were removed primarily in the ion-exchange chromatography step. Final purification was accomplished by gel chromatography (Fig. 2). The nucleic acids removed in the latter step were centrifuged under the same conditions as the proteoglycans, and the banding positions are indicated in Figs. 3 and 5. In CsCl the nucleic acids banded sharply at a density of 1.70, i.e. between the PG-I and PG-II peaks, and, if they had been present in large amounts, they would have been a major contaminant of fraction PG-I but not of fraction PG-II. In Cs_2SO_4 the nucleic acids banded sharply at a density of 1.44, again between the major PG-I and PG-II peaks. In this case most of the proteoglycans would have been obtained free of DNA by such a step, but the shoulder on the PG-I peak would have been contaminated with nucleic acids. Although column chromatography is an important step in preparing the proteoglycans free from nucleic acids, 80% of the DNA would have been removed directly by isopycnic centrifugation in Cs_2SO_4 .

As isopycnic centrifugations in Cs_2SO_4 may give rise to a separation of proteoglycans according to the uronate composition of their side chains, given that other parameters are fairly invariable, this technique should be useful in the study of complex mixtures of proteoglycans that are found in different connective tissues and are secreted by fibroblast cultures (Carlstedt *et al.*, 1981).

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