Properties of Fractionated Chondroitin Sulphate from Ox Nasal Septa

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1. Chondroitin sulphate was isolated from bovine nasal septa by precipitation with cetylpyridinium chloride after digestion of the tissue with papain. 2. The material was divided into two portions, one of which was partially degraded with testicular hyaluronidase. 3. Untreated and hyaluronidase-digested material were fractionated into a total of eleven subfractions by gel chromatography on Sephadex G-200 and Sephadex G-100 respectively. 4. Chemical analyses indicated that the composition of all the fractions was similar to that of chondroitin sulphate. However, electrophoresis revealed a charge-inhomogeneity in the low-molecular-weight fractions obtained after hyaluronidase digestion. 5. The physicochemical properties of the subfractions were investigated by sedimentation-velocity, diffusion and sedimentation-equilibrium studies, osmometry, viscometry and gel chromatography. The individual fractions were essentially monodisperse and showed molecular weights ranging from 2400 to 36000. 6. The relationship between the intrinsic viscosity and the molecular weight was $[\eta] = 5.0 \times 10^{-6} \times M^{1.14}$, indicating that the chondroitin sulphate molecules assume a shape intermediate between that of a random coil and a stiff rod. 7. The relationship between the sedimentation constant and the molecular weight ($>10^4$) was $s_{20,w}^0 = 2.3 \times 10^{-2} \times M^{0.44}$.

Chondroitin sulphate, like most sulphated glycosaminoglycans, occurs *in vivo* covalently bound to protein by a characteristic linkage [for references see e.g., Rodén (1968), Barrett (1968) and Schubert & Hamerman (1968)]. The chondroitin sulphate proteoglycan is abundant in most cartilaginous tissues, but is also found elsewhere (Brimacombe & Webber, 1964). Single chondroitin sulphate peptides are readily liberated from the tissues by digestion with papain, whereas polysaccharide chains free of protein may be obtained by treatment with alkali (Luscombe & Phelps, 1967; Anderson, Hoffman & Meyer, 1965). The free polysaccharide contains *N*-acetylgalactosamine, glucuronic acid and, generally, sulphate in equimolar quantities (Brimacombe & Webber, 1964).

Chondroitin sulphate is disperse with regard to molecular weight and has a relatively extended molecular configuration. These properties, as well as the polyanionic character of the molecule, result in a marked non-ideality of solutions of the polysaccharide. In most previous studies on the molecular weight of chondroitin sulphate, polydisperse preparations were employed yielding average molecular-weight values that varied according to the method of analysis. Although chondroitin sulphate has been separated into subfractions with different average molecular weights (Mathews,

1956), the molecular-weight values obtained by different methods for essentially monodisperse polysaccharide fractions have not been compared. In the present investigation such fractions have been obtained by gel chromatography, a technique that has been previously shown to separate molecules according to size (for references see Laurent, Öbrink, Hellsing & Wasteson, 1969). The physicochemical properties of these fractions have been investigated by several independent methods. The ultimate aim of this investigation was to produce fractions of chondroitin sulphate of known molecular weights, for use as standards in the determination of molecular weights of unknown samples of chondroitin sulphate by gel chromatography. This method has been outlined previously (Wasteson, 1969).

MATERIALS AND CHEMICAL METHODS

A preparation of chondroitin sulphate containing approximately equimolar amounts of galactosamine and sulphate and a preparation of hyaluronic acid were kindly supplied by Dr E. Wessler, and heparin, prepared from a commercial product (Vitrum AB, Stockholm, Sweden) by precipitation with cetylpyridinium chloride (Lindahl, Cifonelli, Lindahl & Rodén, 1965), was provided by Dr U. Lindahl at our Institute. Dermatan sulphate from pig skin was generously given by Dr L. Rodén,

Departments of Pediatrics and Biochemistry, University of Chicago, Chicago, Ill., U.S.A. Three homogeneous and well-characterized Ficoll (a highly cross-linked polysucrose) fractions, nos. XIII, XV and XVI with Stokes radii of 49.0, 34.5 and 26.4 Å respectively, were available in our laboratory (Laurent & Granath, 1967). A high-molecular-weight dextran fraction (FDR 1197, $\bar{M}_w = 12 \times 10^6$) was kindly supplied by Pharmacia, Uppsala, Sweden. Dextran gels, Sephadex G-200, lot nos. 8987 and 1003, and Sephadex G-100, lot no. 9262, were obtained from Pharmacia. Testicular hyaluronidase (EC 3.2.1.35; 14 000 units/mg) was obtained through the courtesy of Dr B. Högborg, AB Leo, Hälsingborg, Sweden. Papain (EC 3.4.4.10) was purchased from E. Merck A.-G., Darmstadt, Germany. Dialysis tubing (23/32 in) was obtained from Union Carbide Corp., Chicago, Ill., U.S.A. Hexuronic acid was determined by a modification of the carbazole reaction (Bitter & Muir, 1962) with glucuronolactone as a standard. Hexosamine was determined by the method of Elson & Morgan as described by Blix (1948). Sulphate was determined as described by Antonopoulos (1962) and nitrogen by a modification of the Kjeldahl method (Ma & Zuazaga, 1942). The galactosamine/glucosamine ratio was estimated by paper chromatography as described by Stoffyn & Jeanloz (1954) and by g.l.c. as described by Radhakrishnamurthy, Dalferes & Berenson (1966). Samples were prepared for g.l.c. by hydrolysis in 4M-HCl at 100°C for 14 h.

The concentration of dextran in column effluents was determined by the anthrone reaction (Scott & Melvin, 1953).

Tritium was measured in a Packard Tri-Carb liquid-scintillation counter.

EXPERIMENTAL AND RESULTS

Preparation of chondroitin sulphate subfractions

Isolation of chondroitin sulphate. Bovine nasal septa, obtained from a slaughterhouse within 1 h of slaughter, were freed of adhering non-cartilaginous tissue, rinsed with 0.9% sodium chloride and sliced. All operations were carried out at 4°C.

Cartilage slices were digested with papain (Fransson & Rodén, 1967) and the liberated polysaccharide was recovered as a cetylpyridinium complex from 0.3M-sodium chloride (Scott, 1960). Sodium chondroitin sulphate was precipitated from a solution of this complex in 2M-sodium chloride by the addition of 3 vol. of ethanol. The precipitate was washed once with ethanol and dried with ether.

Hyaluronidase treatment. Part of the chondroitin sulphate was partially degraded with testicular hyaluronidase. A 1% (w/v) solution of chondroitin sulphate was prepared by dissolving 2 g of polysaccharide in 200 ml of 0.1M-acetate buffer-0.15M-sodium chloride, pH 5.0 (Fransson & Rodén, 1967). The solution was incubated in a water bath at 37°C with 5600 units of testicular hyaluronidase. The reaction was allowed to proceed for 16 h, corresponding to a 50% decrease in the viscosity number, and was then interrupted by heating

the reaction mixture on a boiling-water bath for 10 min.

Fractionation of chondroitin sulphate by gel chromatography. Fractionation of untreated chondroitin sulphate was performed on a column (5.0 cm × 95 cm) of Sephadex G-200 (Laurent & Granath, 1967). The column was eluted by upward flow with 0.2M-sodium chloride by means of a peristaltic pump at a rate of about 3 ml · cm⁻² · h⁻¹. The void volume, V_0 , and the total volume, V_t , of the column, were determined by chromatography of 50 mg of dextran (FDR 1197) and 3 μ Ci of tritiated water respectively (Marsden, 1965; Öbrink, Laurent & Rigler, 1967). Samples of 2 g of chondroitin sulphate, dissolved in 20 ml of 0.2M-sodium chloride, were introduced into the column followed by 20 ml of 15% (w/v) sodium chloride to prevent convective flow. The eluate was collected in fractions of about 15 ml which were analysed for uronic acid and the effluent volume was determined gravimetrically.

Chromatography of chondroitin sulphate on the Sephadex G-200 column produced the chromatogram shown in Fig. 1(a). The eluate was divided into six fractions as indicated by the arrows in the figure; the corresponding fractions from

Fig. 1. Preparative gel chromatography (a) on Sephadex G-200 of chondroitin sulphate and (b) on Sephadex G-100 of hyaluronidase-treated chondroitin sulphate. The eluates were analysed for hexuronic acid and divided into fractions as indicated by the arrows.

Table 1. *Chemical analysis of chondroitin sulphate fractions*

The values are expressed as percentage of dry weight. The molar amounts relative to glucuronic acid are given in parentheses.

Fraction no.	Hexosamine	Glucuronic acid	Nitrogen	Sulphur
1	30.8 (0.91)	37.0 (1.00)	2.9 (1.10)	6.2 (1.02)
2	29.9 (0.86)	38.1 (1.00)	2.9 (1.05)	6.0 (0.95)
3	29.5 (0.85)	37.9 (1.00)	3.0 (1.10)	5.9 (0.95)
4	28.3 (0.91)	33.9 (1.00)	2.8 (1.15)	5.5 (0.98)
5	27.2 (0.99)	30.1 (1.00)	2.6 (1.18)	5.3 (1.07)
6	27.3 (0.95)	31.5 (1.00)	—	—
7	32.1 (1.04)	33.8 (1.00)	2.6 (1.07)	5.5 (0.98)
8	31.9 (1.05)	33.4 (1.00)	2.6 (1.09)	5.3 (0.96)
9	31.4 (1.03)	32.9 (1.00)	2.5 (1.05)	5.3 (0.97)
10	32.2 (1.06)	33.2 (1.00)	2.4 (1.00)	5.2 (0.96)

three separate runs were pooled. These fractions, denoted 1–6, were dialysed several times against water and freeze-dried.

The hyaluronidase-treated material was concentrated *in vacuo* to a volume of about 20 ml and was then chromatographed on a column (5.0 cm × 100 cm) of Sephadex G-100, under the conditions described above. The effluent fractions were combined into five fractions (Fig. 1b), which were concentrated in a rotary evaporator to a volume of approx. 40 ml and desalted afterwards on a column (2.0 cm × 70 cm) of Sephadex G-25. The salt-free fractions, denoted 7–11, were stored in the freeze-dried form.

Chemical properties of chondroitin sulphate fractions. The chemical analyses of the chondroitin sulphate fractions (Table 1) were in good agreement with data given in the literature for the polysaccharide. Galactosamine was the only amino sugar detected by the method of Stoffyn & Jeanloz (1954) or by g.l.c.

The polysaccharide fractions were further examined by electrophoresis in 0.1M-barium acetate (Wessler, 1968), and this technique revealed the presence of chondroitin sulphate only. A distinct separation of chondroitin sulphate from the reference compounds, heparin, hyaluronic acid and dermatan sulphate, was observed. The degree of sulphation was estimated by electrophoresis in 0.1M-hydrochloric acid (Wessler, 1970). The chondroitin sulphate fractions 1–5, 7 and 8 showed migration rates identical with that of the reference sample of chondroitin sulphate, containing one sulphate residue per disaccharide unit. Fraction 6 migrated somewhat slower than the reference sample, indicating some undersulphation of this fraction. Fractions 9–11 contained components migrating both slower and faster than the reference. This finding is discussed below.

Physicochemical analyses

Analytical gel chromatography. Analytical gel chromatography was carried out on a column

Fig. 2. Analytical gel chromatography on Sephadex G-200 of chondroitin sulphate fractions 1–6 (a) and 7–11 (b). The eluates were analysed for hexuronic acid. (a) Fraction 1 (■); fraction 2 (□); fraction 3 (▲) Fraction 4 (△); fraction 5 (●); fraction 6 (○). (b) Fraction 7 (■); fraction 8 (□); fraction 9 (▲); fraction 10 (△); fraction 11 (○).

(0.8 cm × 63.5 cm) of Sephadex G-200, equilibrated with 0.2 M-sodium chloride. Samples of 200 μg of chondroitin sulphate or 1 mg of Ficoll, dissolved in 200 μl of 0.2 M-sodium chloride, were applied to the column and eluted with 0.2 M-sodium chloride at a flow rate of 3–5 ml · cm⁻² · h⁻¹. Effluent fractions of about 1 ml were collected and weighed, and analysed by the carbazole or by the anthrone reactions. The void volume, V_0 , and the total volume, V_t , of the column were determined by chromatography of 1 mg of dextran and 0.1 μCi of tritiated water respectively.

Chromatography of the chondroitin sulphate fractions 1–11 produced the chromatograms shown in Fig. 2. Narrow and symmetrical peaks were obtained, indicating a relatively uniform size of the polymer chains within each fraction. The elution volume, defined as the position of the maximum of each eluted peak, was determined in two separate experiments. This elution volume was related to V_0 and V_t , giving the available volume fraction, K_{av} , for the solute in the gel phase, by the equation of Laurent & Killander (1964). The K_{av} values are listed in Table 2. The relationship between K_{av} and the Stokes radius (r_s) of a compound is (Laurent & Killander, 1964):

$$K_{av} = \exp[-\Pi \cdot L(r_r + r_s)^2] \quad (1)$$

where L is the total length of the gel fibres per unit volume and r_r is the radius of the fibres. Chromatography of the Ficoll fractions XIII, XV and XVI gave K_{av} values of 0.20, 0.43 and 0.59 respectively, and these values together with the Stokes radii of the fractions (see the Materials and Chemical Methods section) established the values of L and r_r . Eqn. (1) could then be used directly for calculating r_s for each chondroitin sulphate fraction (Table 2). Apparent diffusion constants, D_{app} , were calculated from the Stokes radii by the equation given by Sutherland (1905):

$$D_{app} = \frac{kT}{6\Pi\eta_0 r_s} \quad (2)$$

where k is Boltzmann's constant, T is the absolute temperature and η_0 is the viscosity of the solvent. The calculated diffusion constants are also listed in Table 2.

Apparent molecular weights, $\bar{M}_{w(s/D_{app})}$, were calculated from the apparent diffusion constants and sedimentation constants (see below) (Table 2) from Svedberg's equation:

$$M_w = \frac{sRT}{D(1 - \bar{v}\rho)} \quad (3)$$

where R is the gas constant, $\bar{v}$ is the partial specific volume and ρ is the density of the solvent (Svedberg & Pedersen, 1940).

Table 2. *Physicochemical parameters of chondroitin sulphate fractions*

For explanation of the symbols see the text.

Fraction no.	K_{av} on Sephadex G-200	r_s (Å)	$10^{13} \times s_{20,w}^0$ (s)	$10^7 \times D_{20,w}^0$ (cm ² /s)	$10^7 \times D_{20,w}^0$ (cm ² /s)	$10^7 \times D_{app}$ (cm ² /s)	$[\eta]$ (100 ml/g)	$10^{-6} \times \beta$	$\bar{M}_{w(s/D_{20,w}^0)}$	$\bar{M}_{w(s/D_{app})}$	$\bar{M}_{w(eq.)}$	$\bar{M}_n$
1	0.11	58.5	2.43	2.9	3.64	3.64	0.82	2.8	44500	35600	36000	42100
2*	0.20	49.5	2.00	3.9	4.30	4.30	0.55	2.5	27200	24700	26000	34100
3	0.28	43.5	1.75	4.3	4.90	4.90	0.40	2.4	21200	19000	19700	20500
4	0.34	39.5	1.68	(4.3)	5.39	5.39	0.32	2.4	(20700)	16500	16400	15600
5	0.41	35.0	1.57	(5.4)	6.08	6.08	0.30	2.4	(15400)	13700	14100	14400
6	0.47	32.0	1.39	(6.6)	6.65	6.65	—	—	(11200)	11100	—	12200
7	0.30	42.0	1.75	—	5.07	5.07	0.38	2.3	—	18300	22000	—
8	0.45	33.0	1.50	—	6.45	6.45	0.26	2.4	—	12400	12300	—
9	0.61	25.0	1.33	—	8.52	8.52	0.15	2.2	—	8300	8800	—
10	0.74	18.0	1.24	—	11.8	11.8	—	—	—	5600	4100	—
11	0.84	12.5	0.82	—	17	17	—	—	—	2600	2200	—

* This fraction was also analysed by light-scattering experiments in a Sofica PGD 42000 photometer. Measurements were performed on solutions (1–4 mg/ml) in 0.4 M-NaCl at room temperature. By using a value of 0.139 for the refractive index increment at this ionic strength (B. Öbrink & Å. Wasteson, unpublished work), a weight-average molecular weight of 25000 was obtained.

Ultracentrifugation. Ultracentrifugation was performed in a Spinco model E ultracentrifuge. All experiments were carried out at 20.0°C in an AnD rotor. Solutions of chondroitin sulphate in 0.2M-sodium chloride, containing 10–15mg of polysaccharide/ml, were dialysed against the solvent and diluted with the diffusate to yield solutions of 1–15mg of polysaccharide/ml. Final concentrations were calculated from determinations of hexuronic acid.

Partial specific volume. The value 0.54ml/g for the partial specific volume, $\bar{v}$, of chondroitin sulphate (Elias & Patat, 1960; Luscombe & Phelps, 1967) was used for calculating molecular weight.

Sedimentation. Sedimentation coefficients were determined in a 12mm capillary-type synthetic-boundary cell. Sedimentations were carried out at 59780 rev./min. The schlieren patterns were registered on photographic plates and analysed in a microcomparator. Sedimentation coefficients ($s_{20,w}$) were calculated as described by Schachman (1957). The reciprocal of the sedimentation coefficients of fractions 1–6 and the sedimentation coefficients of fractions 7–11 respectively are shown in Fig. 3 as a function of the concentration. A marked concentration dependence was evident, especially for the fast-sedimenting species. For fractions 1–6 the extrapolation to zero concentration was guided by linear extrapolation of the reciprocal of the sedimentation coefficients (Schachman, 1957). The calculated $s_{20,w}^0$ values are recorded in Table 2.

Diffusion. Diffusion coefficients, $D_{20,w}$, were determined in the ultracentrifuge in a 12mm double-sector synthetic-boundary cell, which was run at 4908 rev./min. The height and the area of the schlieren peaks were measured as a function of time and the results were treated as described by Van Holde (1960). The measurements on the schlieren recordings were difficult to perform, mainly owing to the rapid diffusion; this was true particularly for the low-molecular-weight fractions, where the peaks diffused rapidly. The diffusion coefficients obtained for fractions 1–3 are shown in Fig. 4 as a function of concentration. The values extrapolated to infinite dilution, $D_{20,w}^0$, are given in Table 2. Fractions 4–6 are shown in parentheses in Table 2, since the extrapolations were uncertain. Sedimentation and diffusion values were used to calculate weight-average molecular weights, $\bar{M}_{w(s/D_{20,w}^0)}$ from Svedberg's equation (Svedberg & Pedersen, 1940) (Table 2).

Sedimentation equilibrium. Sedimentation-equilibrium studies were carried out with a 12mm double-sector cell by using the Rayleigh interference optical system. The 'conventional' method (fractions 2–5 and 9–11) and 'meniscus depletion' method (fractions 1, 7 and 8) were applied at 15220–47660 and 27690–52640 rev./min respective-

Fig. 3. Sedimentation-velocity experiments on chondroitin sulphate fractions. The reciprocals of the sedimentation coefficients ($1/s$) of fractions 1–6 [(a) 1 (■); 2 (□); 3 (▲); 4 (△); 5 (●); 6 (○)] and the sedimentation coefficients (s) of fractions 7–11 [(b) 7 (■); 8 (□); 9 (▲); 10 (△); 11 (●)] were recorded at different concentrations of polysaccharide (g/ml).

ly (for references see Chervenka, 1969). Satisfactory equilibrium, i.e. no fringe displacement on repeated registration, was established in 30–40 h. The separate determinations of solute concentration, required in the 'low-speed' experiments, were performed with a 12mm double-sector synthetic-boundary cell. The conventional equations for the calculation of weight-average molecular weights ($\bar{M}_{w(eq.)}$) were used (for references see e.g. Van Holde, 1967). The calculated values at different concentrations are recorded

Fig. 4. Diffusion experiments on chondroitin sulphate fractions. The diffusion coefficients (cm^2/s) of fractions 1–3 [1 (■); 2 (□); 3 (▲)] are plotted against concn. of polysaccharide (g/ml).

in Fig. 5. The plots were extrapolated to zero concentration as shown. The $\bar{M}_w$ values corresponding to infinite dilution are given in Table 2 ($\bar{M}_{w(\text{eq.})}$).

Osmometry. Number-average molecular weights ($\bar{M}_n$) were determined in an electronic high-speed membrane osmometer (Hewlett-Packard model 501, F & M Scientific Corp., Avondale, Pa., U.S.A.) (Armstrong, 1968; Paglini, 1968). The membrane consisted of dialysis tubing, which had previously been washed with 10% (v/v) acetic acid, soaked in water at 80°C for 1 h and equilibrated with 0.2M-sodium chloride. This material was found to be superior to the membrane supplied by the manufacturer of the instrument; however, relatively long equilibration times were required. Solutions of chondroitin sulphate (approx. 10mg/ml) in 0.2M-sodium chloride were dialysed against the solvent and diluted with appropriate amounts of the diffusate to yield solutions of 1–10mg of polysaccharide/ml. Final concentrations were calculated from hexuronic acid analyses. The solutions of chondroitin sulphate were analysed in the osmometer at 25.0°C with 0.2M-sodium chloride as a solvent. Equilibrium was attained in the instrument 3–5 h after introduction of the sample.

The osmotic pressure (Π) divided by the concentration (c) was plotted against the concentration for each sample and extrapolated to infinite dilution; the relationship between Π/c and c is linear (Fig. 6). From the intercept obtained after extrapolation to zero concentration, $\bar{M}_n$ may be calculated from van't Hoff's equation (Bonnar, Dimbat & Stross, 1958). The $\bar{M}_n$ values are given in Table 2.

Viscometry. Viscometry was carried out in Ubbelohde viscometers (Van Wazer, Lyons, Kim & Colwell, 1963) at 25.0°C . The kinetic errors in the

Fig. 5. Sedimentation-equilibrium experiments on chondroitin sulphate fractions. The weight-average molecular weights ($\bar{M}_{w(\text{eq.})}$) of fractions 1–5 [(a) 1 (■); 2 (□); 3 (▲); 4 (△); 5 (●)] and 7–11 [(b) 7 (■); 8 (□); 9 (▲); 10 (△); 11 (●)] were recorded at different concentrations of polysaccharide (g/ml).

viscometers were negligible. Solutions of chondroitin sulphate in 0.2M-sodium chloride, containing 1–5mg of polysaccharide/ml, were prepared. Dust was removed from the solutions by careful filtration through Celite. Final concentrations were determined by the carbazole reaction. Intrinsic viscosities $[\eta]$ were calculated according to the conventional equation:

$$[\eta] = \lim_{c \rightarrow 0} \left(\frac{\eta}{\eta_0} - 1 \right) / c \quad (4)$$

Fig. 6. Osmometry on chondroitin sulphate fractions: 1 (■); 2 (□); 3 (▲); 4 (△); 5 (●); 6 (○). Osmotic pressures (in cm of water) of fractions 1–6 divided by the concn. of polysaccharide (g/ml) are given for different concentrations.

where η is the viscosity of the solution and c is the concentration, expressed as g/100ml. Intrinsic-viscosity values are given in Table 2.

DISCUSSION

The chemical composition of the polysaccharide fractions appears to be in good agreement with that of chondroitin sulphate. The molar ratios (Table 1) were based on the hexuronic acid rather than on the hexosamine analyses, since it was considered that the former were more reproducible in our hands than were the latter. For some unknown reason the hexosamine values were generally somewhat higher in the hyaluronidase-treated than in the non-treated fractions. The sulphate analyses revealed approximately 1 mol of sulphate/disaccharide unit in all the chondroitin sulphate fractions analysed. This finding applied also to those hyaluronidase-treated fractions that contained chains of different sulphate density, as demonstrated by electrophoresis in 0.1 M-hydrochloric acid.

Numerous papers on the molecular weight of chondroitin sulphate preparations have appeared in the literature and widely different values have been reported. In most of the earlier studies (see e.g. review by Elias & Patat, 1960) relatively high molecular-weight values were found. These values may be accounted for by the mild preparation methods used, which presumably left some of the peptide links between chains intact. By treatment with alkali or digestion with papain the chondroitin sulphate proteoglycan is readily degraded to single

polysaccharide chains and polysaccharide-peptides, respectively (Luscombe & Phelps, 1967). The average molecular weight of the product determined by several independent methods has generally been found to range between 10 000 and 45 000 (Mathews, 1956; Partridge, Davis & Adair, 1961; Buddecke, Kröz & Lanka, 1963; Marler & Davidson, 1965; Luscombe & Phelps, 1967; Greiling, Stuhlsatz & Plagemann, 1967; Hjertquist & Vejlens, 1968; Wasteson, 1969; Lindahl & Wasteson, 1970).

The scatter of the reported molecular-weight values for chondroitin sulphate is probably the expression of an inherent molecular polydispersity. However, it may also reflect methodological difficulties to an extent that is difficult to evaluate.

The present fractionation experiments support the concept of a molecular polydispersity in chondroitin sulphate, since a separation of chains of very different sizes was achieved. The molecular dispersity of the individual subfractions was low compared with that of the unfractionated material, as demonstrated by the narrow and symmetrical peaks in the analytical gel chromatograms and by the physicochemical measurements. The various physicochemical methods applied gave molecular-weight values in the range 10 000–40 000. This range was extended downwards to 2000 by fractionation of partially degraded chondroitin sulphate chains. As Table 2 shows the molecular weights obtained by independent methods are in good agreement. Almost identical $\bar{M}_w$ values were obtained by sedimentation-equilibrium experiments ($\bar{M}_{w(eq.)}$) and by sedimentation-velocity determinations in conjunction with measurements by gel chromatography of apparent diffusion coefficients ($\bar{M}_{w(s/D_{app.})}$). Molecular weight values ($\bar{M}_{w(s/D_{20,w}^0)}$) determined by use of the diffusion coefficients obtained by ultracentrifugation were slightly higher than the $\bar{M}_{w(s/D_{app.})}$ values, indicating that the determination of apparent diffusion coefficients by size measurements in gel chromatography may be superior.

The number-average molecular-weight values ($\bar{M}_n$) were somewhat higher than the $\bar{M}_{w(s/D_{app.})}$ and $\bar{M}_{w(eq.)}$ values, especially for fractions 1 and 2. This finding might be explained by a decrease in the osmotic pressures during the measurements as a result of permeation of solute through the membrane. However, since the osmotic pressure remained stable with time such an explanation seems dubious.

The intrinsic-viscosity values of the various chondroitin sulphate fractions (Table 2) were inserted together with the corresponding molecular-weight data in the modified Staudinger equation:

$$[\eta] = K \times M^\alpha \quad (5)$$

Fig. 7. Relationship between intrinsic viscosity and molecular weight of chondroitin sulphate fractions. The intrinsic viscosities of fractions 1-5 and 7-9 (100 ml/g) are plotted against $\bar{M}_{w(\text{eq.})}$ (●) and $\bar{M}_{w(s/D_{\text{app.}})}$ (○) on a double-logarithmic scale.

Fig. 8. Relationship between sedimentation coefficient and molecular weight of chondroitin sulphate fractions. The $s_{20,w}^0$ values of fractions 1-11 are plotted against $\bar{M}_{w(\text{eq.})}$ (●) and $\bar{M}_{w(s/D_{\text{app.}})}$ (○) on a double-logarithmic scale.

where K and α are constants, characteristic of the system (Tanford, 1961). As Fig. 7, a double-logarithmic plot of $[\eta]$ against molecular weight, shows the present results seem to satisfy the equation. The values of the constants K and α were obtained from the linear function (Fig. 7), calculated by the least-squares method. The K value is 5.0×10^{-6} and the α value is 1.14. When the extreme fractions, nos. 1 and 9 with $[\eta]$ 0.82 and 0.15 respectively, were omitted from the calculations, the values of K and α were 1.7×10^{-5} and 1.01 respectively. Mathews (1956) gives α 0.74, i.e. a lower value. The comparatively high value of α in

the present study indicates that the structure of the chondroitin sulphate chains is intermediate between that of an open coil and a stiff rod; in theory the exponent for a coil should be 0.8 and for a rod 1.8 (Tanford, 1961). Hyaluronic acid with molecular weights $> 100\,000$ has α 0.82 in the same solvent, whereas hyaluronic acid fractions of lower molecular weight show higher values (R. L. Cleland, personal communication).

The relationship between sedimentation coefficient and molecular weight was similarly investigated. A double-logarithmic plot of s against M leads to a slight curve (Fig. 8). In the interval 10000-35000 mol.wt. units the correlation is essentially linear, suggesting the relationship:

$$s_{20,w}^0 = 2.34 \times 10^{-2} \times M^{0.44}$$

As a general test the Mandelkern & Flory (1952) equation was applied to the present physicochemical data. The Mandelkern & Flory parameter β was calculated by the relationship:

$$\beta = \frac{[\eta]^{1/3} \cdot \eta_0 \cdot N_A \cdot s}{M^{2/3} \cdot (1 - \bar{v}\rho)} \quad (6)$$

where N_A is Avogadro's number and the other symbols are the same as those described above. The β values of fractions 1-5 and 7-9 were in the order of magnitude of 2.4×10^6 (Table 2). Since flexible coiling polymers give $\beta = 2.6 \times 10^6$ - 2.7×10^6 (Kurata & Stockmeyer, 1963) the present values are in good agreement with the theoretically expected ones. Previously, β values of 2.1×10^6 and 3.0×10^6 have been recorded for chondroitin sulphate (Mathews, 1956) and hyaluronic acid (R. L. Cleland, personal communication) respectively.

In conclusion, the physicochemical characterization of the chondroitin sulphate preparations in this paper indicates that the molecular weights within each fraction are essentially homogeneous, thereby justifying the use of these preparations for reference purposes.

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