MACROMOLECULAR PROPERTIES OF ISOMERIC CHONDROITIN SULFATES*

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SUMMARY

1. Three isomeric chondroitin sulfates of connective tissue, CS-A, CS-B, and CS-C, are found to be linear polyelectrolytes with similar chain configuration–charge relationships.

2. Clear distinction of these substances as molecular individuals with isomeric repeating units is established by comparison of molecular constants and physical properties and confirmed by infra-red analyses.

3. Molecular weight, intrinsic viscosity, solubility, electrophoretic mobility, and potentiometric titration data are reported.

INTRODUCTION

Three different acid mucopolysaccharides from connective tissue possess disaccharide repeating units (Fig. 1) containing equimolar amounts of hexuronic acid, N-acetyl D-galactosamine and ester sulfate (see review by MEYER *et al.*[1]). These substances, respectively, chondroitin sulfate A (CS-A), chondroitin sulfate B (CS-B) and chondroitin sulfate C (CS-C), differ in solubility, optical rotation, and distribution in

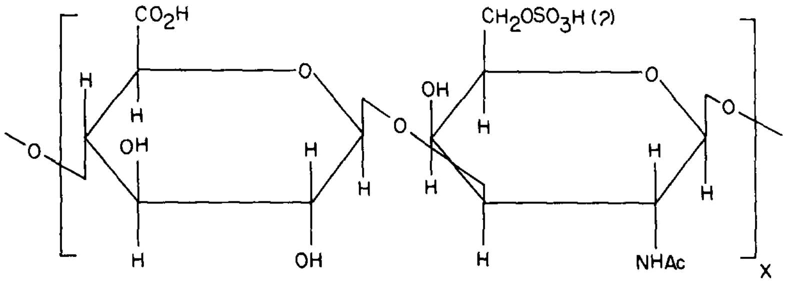

Fig. 1. Repeating disaccharide unit of isomeric chondroitin sulfates A and C. Chondroitin sulfate B contains L-iduronic acid, the C-5 epimer of D-glucuronic acid shown above. The position of the aminidic linkage to uronic acid is uncertain. It is likely that the sulfate group has the C-6 position shown for CS-C and is in the C-4 position in CS-A and CS-B[20].

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connective tissues. Further, CS-A and CS-C both contain D-glucuronic acid and are degraded by testicular hyaluronidase; CS-B contains L-iduronic acid, is resistant to testicular hyaluronidase, and possesses anti-coagulant properties[2].

It has been suggested[1] that CS-A and CS-C either differ in some detail of the repeating unit, or, possess identical repeating units and polymer structure and differ only in degree of polymerization. BERNARDI, HAPPEY AND NAYLOR[3] have recently offered evidence in support of the "hypothesis that chondroitin sulfates A and C are only two fractions of the same polydisperse substance". The purpose of this communication is to present physical-chemical data which permit clear distinction of CS-A and CS-C as molecular individuals. Some specific relationships between macromolecular structure and properties for the sodium salts of the three polysaccharides are also reported.

MATERIALS AND METHODS

Preparations of CS-A from bovine nasal septa are those previously described[4,5]. CS-B, a sample of β-heparin (beef lung), lot No. RO1-2232/715 obtained from F. Hoffmann-La Roche and Co., Basle, through the courtesy of Dr. A. WINTERSTEIN, has been described[6]. CS-C as the calcium salt, kindly furnished by Dr. KARL MEYER, had been obtained from a human chordoma. Analyses for this material have been reported[1]. It was converted by ion exchange to the sodium salt. Full investigation of CS-C was limited by the small amount available.

Methods for the determination of osmotic pressure molecular weight[4], intrinsic viscosity[4], viscosity function in water[5], potentiometric titrations[5], and light scattering molecular weight[7] of acid mucopolysaccharides are those previously described. It was necessary to clarify CS-B solutions prior to light scattering measurements by centrifugation at 80,000 × *g* followed by filtration in order to remove traces of high molecular weight impurities. Depolarization and dissymmetry ($\sim$1.02) of solutions of CS-B were then negligible. However, minor corrections for a persistent fluorescence were required. All polysaccharide concentrations are based on an "anhydrous" content of each preparation, determined by differential refractometry[7]. The refractive increment for 100 % "anhydrous" content was determined as 0.166 when the solvent is 0.15 M sodium phosphate buffer containing 0.2 M NaCl, and as 0.181 when water is the solvent.

Fractionation of CS-B was carried out as follows. A known volume of 95 % ethanol (measured at 25°) was added to a solution containing 5.0 mg polysaccharide/ml of 0.5 % aqueous sodium acetate. The flask containing the clear solution was placed in a bath at 0.5° and stirred continuously for 20–24 h. The resulting precipitate was removed by centrifugation at 0.5°, washed with anhydrous ethanol followed by anhydrous ether and dried *in vacuo* over $CaCl_2$. Further additions of ethanol were made to successive supernatants and each fraction removed similarly. Electrophoresis was carried out with a Perkin-Elmer Tiselius apparatus at 22° and conductance measurements of polysaccharide solutions at 25°.

RESULTS AND DISCUSSION

Fractionation of CS-B

Results of fractionation of 10 g of CS-B are shown in Table I. The absence of variation in analytical values within the probable error of colorimetric procedures (± 8 %) indicates the essential chemical homogeneity of the original material. The intrinsic viscosity values indicate a moderate heterogeneity of molecular weight with the bulk of the material falling within a relatively narrow molecular size range.

TABLE I

FRACTIONATION OF β-HEPARIN (CS-B)

Fraction	*Weight* g	*Vol. ratio* *alc./water*	$[\eta]$	*N* %	*Uronic acid** %	*Hexosamine*** %	*Anhydrous content* %	*Ester sulfate**** % *S*
Original (o)	10.00		0.46	2.4	17.3	25.0	78	4.9
F1	0.08	1.10		2.4	19.4	21.0		
F2A	1.03	1.15	0.57	2.4	17.6	23.2	79	
F2B	0.42	—						
F3A	1.50	1.20	0.57	2.4	15.6	24.4	78	4.9
F3B	2.28	—	0.53				75	
F4	2.09	1.35	0.50				78	
F5	0.82	1.50	0.49				82	
F6	1.03	1.70	0.34	2.4	18.2	23.8	78	4.8
F7	0.42	2.50	0.15	2.6	16.8	22.6	78	

* By a colorimetric procedure using carbazole[8].

** By a modified ELSON-MORGAN[9] procedure.

*** By a spectrophotometric procedure[10] after 2 h hydrolysis at 100° in 2 *N* HCl.

Molecular constants

Physical constants for representative samples of the three types of polysaccharide are shown in Table II. Intrinsic viscosity and molecular weight for CS-A are related by the experimentally determined relation[7]: $[\eta] = 3.1 \cdot 10^{-4} M_W^{0.74}$, which also applies to the molecular parameters of fraction F3A. Thus, it is possible that CS-B, like CS-A, is a linear, unbranched polymer. Molecular constants (except rotation) are similar for CS-C and for CS-4, or CS3E2A, preparations obtained by mild extraction methods. If allowance is made for molecular heterogeneity, greater for CS-4 than for CS3E2A, it is possible that CS-C and CS-A also have similar chain polymer structures. The difference in rotation between fractions F3A and F6 is mainly due to the presence of about 20 % CS-A (detected enzymically) in F6.

TABLE II

PHYSICAL CONSTANT OF ACID MUCOPOLYSACCHARIDES

Preparation	*Type*	$[\eta]$	M_N	M_W	$[\alpha]_D^{25}$
CS13F	A	0.37	10,000	17,000	
CS-1	A	0.51	13,800	18,000	—35
CS-4	A	0.90	24,600	48,000	—37
CS3E2A	A	0.91	40,000	50,000	
F3A	B	0.57	15,500	22,000	—76
F6	B	0.34	10,000		—59
CS-C	C	0.84	35,000		—19

Viscosity function in water

Dilution of solutions of flexible chain polyelectrolytes in pure water leads to an increase in reduced viscosity (η_{sp}/C) due to an uncoiling of the molecular chain concomitant with an increase in electrostatic charge of the polyion. CS-A, in common with other polyelectrolytes which possess a linear structure, yields data which fit[4] a relation due to FUOSS

$$\eta_{sp}/C = A/(1 + BC^{1/2}) + D \qquad (1)$$

where A, B, and D are empirical constants and C is the concentration in g/100 ml. Viscosity functions for CS-B preparations and for CS-C, shown in Fig. 2, fit eqn. (1) well.

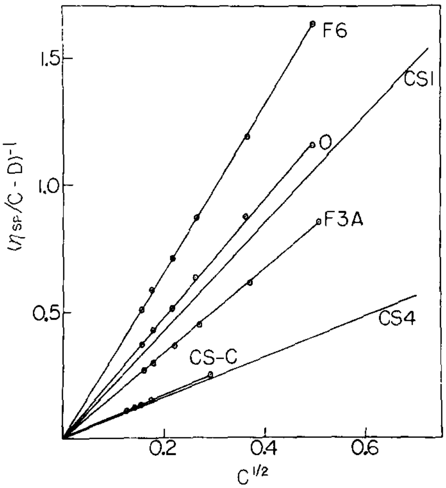

Fig. 2. Viscosity functions in water of isomeric chondroitin sulfates.

The slopes (B/A) of these functions may be broadly interpreted[5] as inversely proportional to $(\eta_{sp}/C)_0$, defined as the intrinsic viscosity of the polyelectrolyte molecule at infinite dilution in water (*i.e.* zero counterion concentration) and hence at near full chain extension. If $[\eta]$ and $(\eta_{sp}/C)_0$ for each preparation are taken as exponential functions of the average molecular weight $[\eta] = KM^a$; $(\eta_{sp}/C)_0 = K'M^{a'}$), these quantities will be related and should fit the expression

$$\log (B/A) = b \log [\eta] + c \qquad (2)$$

where b and c are empirical constants. The validity of eqn. (2) is indicated by the good agreement obtained between experimental values of B/A and those values calculated from eqn. (2) as shown in Table III. It thus appears that all three types of polysaccharide are linear polyelectrolytes with similar chain configuration–charge relationships.

Solubility

Solubilities of each type of polysaccharide are compared for various ethanol concentrations in Table IV. CS-B shows a lower solubility than either CS-A or CS-C, especially evident at an ethanol–water ratio of 1.50. At an ethanol–water ratio of

1.70, CS-C, though of higher average molecular weight, has a greater solubility than CS-1 (type A).

TABLE III

CONSTANTS OF POLYELECTROLYTE EQUATION

Preparation	*Type*	$[\eta]$	*D*	*B/A exper.*	*B/A* calc.*
F6	B	0.34	0.46	3.28	3.55
CS-B (o)	B	0.46	0.55	2.34	2.27
CS-1	A	0.51	0.55	2.12	1.95
F3A	B	0.57	0.62	1.68	1.66
CS-C	C	0.84	1.35	0.88	0.93
CS-4	A	0.90	0.87	0.84	0.84

* Calculated from the empirical relation: $\log (B/A) = -1.49 \log [\eta] - 0.145$.

TABLE IV

PERCENT PRECIPITATION OF MUCOPOLYSACCHARIDES AT 0.5° AND VARIOUS ETHANOL RATIOS*

ml 95% EtOH/ml H_2O	*1.35*	*1.50*	*1.70*	*1.90*
CS-B (o)	70	80	90	>90
CS-1	0	<5	80	90
CS-4	0	<5		>94
CS-C	0	0	20	70

* Initial concentration is 5.0 mg mucopolysaccharide (near 80% anhydrous)/ml water containing 0.5 % sodium acetate.

Potentiometric analysis

The titration curve of a polymeric carboxylic acid may be generally described by the empirical equation

$$\mathrm{pH} = \mathrm{p}K - n \log \frac{1-\alpha}{\alpha} \tag{3}$$

where α denotes degree of ionization of the acid (see review[11]). However, both empirical constants, pK and n, vary with ionic strength. A theoretical expression for the intrinsic ionization constant, pK_0, has been derived which includes the electrostatic potential (ψ_0) of the polyion.

$$\mathrm{pH} = \mathrm{p}K_0 - \log \frac{1-\alpha}{\alpha} + 0.4343 \frac{\varepsilon\psi_0}{kT} \tag{4}$$

Further, ψ_0 has been identified with the differential of the electrostatic energy F_e to the charge of the ionized macromolecule. In particular, for highly stretched polycarboxylic polysaccharides, KATCHALSKI *et al.*[12] have obtained the following expression for the potential

$$\psi_0 = \frac{1}{\varepsilon}\frac{\delta F_e}{\delta v} = \frac{2\alpha\varepsilon}{Dbj} \ln \left(1 + \frac{6}{\varkappa Sb}\right) \tag{5}$$

where j is the distance between carboxylic groups in number of monomeric units;

b is the length of repeating monomeric unit; $\varkappa$ is the inverse DEBYE radius $= 0.327 \cdot 10^8 \, I^{1/2}$, where I is the ionic strength; s is the number of monomeric units per statistical element of the polymer chain; D is the dielectric constant.

KATCHALSKI[13] has suggested that eqn. (5) may be applied to CS-A by replacing α with $(1 + \alpha)$. This is necessary since the sulfate groups of CS-A are fully dissociated in the titration range of the carboxyl groups. Calculations of pK_0 from titration data are therefore based on the complete relation

$$\mathrm{pH} = \mathrm{p}K_0 - \log \frac{1-\alpha}{\alpha} + \frac{2\,\varepsilon^2\,(1+\alpha)}{DkTjb} \log\left(1 + \frac{6}{\varkappa S b}\right) \qquad (6)$$

with $D = 80$, $kT = 4.1 \cdot 10^{-14}$ erg, $b = 5.15 \cdot 10^{-8}$ cm, $\varepsilon^2 = 23 \cdot 10^{-20}$ (e.s.u.)^2, $j = 2$. The value of S is assumed by analogy to be the same for CS-A and for pectic acid where it is 27.

Potentiometric data for two fractions of CS-B are shown in Figs. 3 and 4. Calculated constants appear in Table V. Values of pK and of pK_0 which were calculated for the titrations done at a nearly constant μ of 0.1, are larger for CS-B than for CS-A. The values of pK_0, however, will reflect any error in the theoretical calculation of ψ_0.

Surface potential and net charge from electrophoresis

The success obtained in the calculation of ψ_0 from electrophoretic measurements on the polymethacrylate ion[12] suggested the application of a similar procedure to the chondroitin sulfates. GORIN's[14] values for the C factor for long thin cylinders of

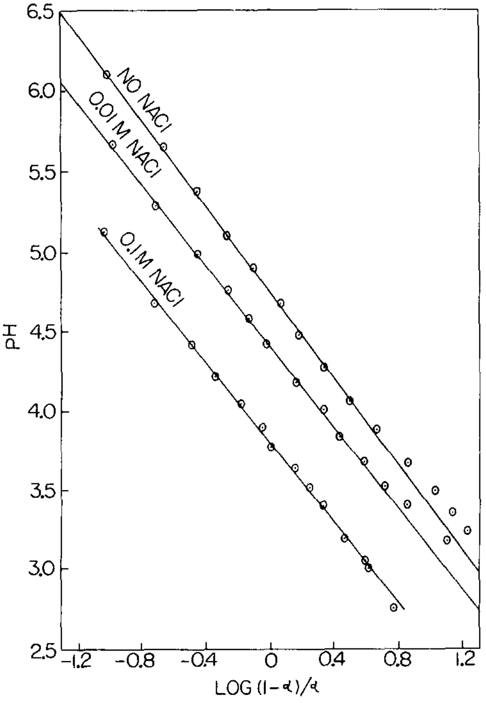

Fig. 3. Potentiometric data for CS-B fraction F3A.

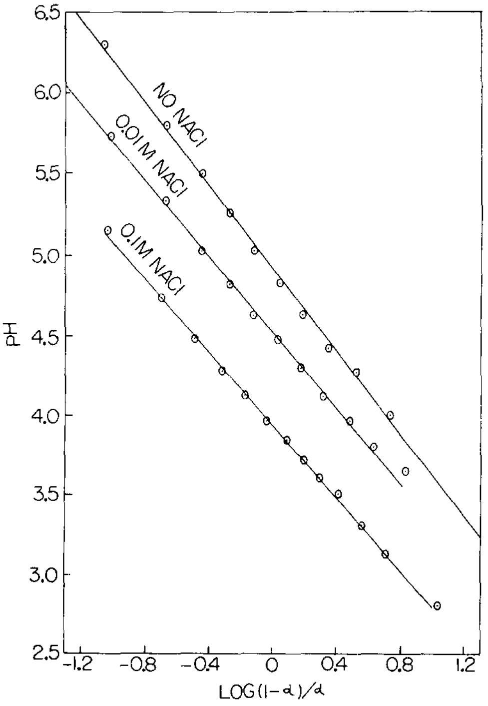

Fig. 4. Potentiometric data for CS-B fraction F6.

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TABLE V

POTENTIOMETRIC TITRATION CONSTANTS

Sample	*NaCl molarity*	*pK**	*n**	*pK₀***
CS1	0	4.41	1.33	
CS4	0	4.34	1.36	
F3A	0	4.92	1.30	
F6	0	4.74	1.34	
CS1	0.01	3.97	1.21	
F3A	0.01	4.52	1.18	
F6	0.01	4.40	1.27	
CS1	0.1	3.35	1.40	3.07 ± 0.08
CS4	0.1	3.38	1.32	3.06 ± 0.06
F3A	0.1	3.93	1.14	3.63 ± 0.02
F6	0.1	3.82	1.25	3.49 ± 0.07

* Calculated from the empirical relation $\mathrm{pH} = \mathrm{p}K - n \log (1-\alpha)/\alpha$.

** Calculated from the theoretical equation $\mathrm{pH} = \mathrm{p}K_0 - \log (1-\alpha)/\alpha + 0.207\,(1+\alpha)$, applicable to 0.1 M NaCl solutions.

3A radius oriented at random at 0.1 ionic strength are used with the well known electrophoretic equation

$$\psi_0 = 300\, C\, \mu\eta/D \tag{7}$$

where μ is the electrophoretic mobility and η and D are, respectively, the viscosity and dielectric constant of the solvent. At pH 7, ψ_0 is calculated from descending mobilities, as $-1.8\cdot 10^4$ V (e.s.u.), corresponding to 0.91 for 0.43 $\varepsilon\psi_0/kT$, or twice the value calculated for the third term on the right of eqn. (6).

This discrepancy is not unexpected in view of the uncertainty in use of the bulk dielectric constant and in the approximate nature of the various molecular parameters. Further, since $\varepsilon\psi_0/kT$ is large, the Debye-Hückel approximations, used in the derivation of eqns. (5) and (7) have only limited justification. The high potentials suggest that counter-ion immobilization occurs. This conclusion is supported by calculations of the net charge of the polyion based on GORIN's[13] equations for cylinders. Approximately 50 % binding of counter-ions is indicated. Comparison of calculated and experimental mobilities in Table VI shows the expected dependence of ψ_0 on $(1 + \alpha)$. The deviations from linearity are apparently not due to changes in molecular frictional properties since intrinsic viscosity is independent of pH in the range studied. This may, however, reflect a variation in binding of counterions with α.

The use of electrophoretic potentials in eqn. (4) leads to values of pK_0 about 0.3 units lower than those of Table V. In either case, the values are not unreasonable when compared to the value of 3.33 for the pK_0 glucuronic acid[16]. Although the absolute values of pK_0 are uncertain, a difference between pK_0 values for CS-A and CS-B of about 0.5 unit seems experimentally established.

The strength of the carboxyl group can be less equivocally determined in a polymer which does not contain ester sulfate. Sodium hyaluronate, from Group A streptococcus, has a repeating disaccharide unit composed of glucuronic acid and acetylglucosamine. The pK_0 of the carboxyl group can in this case be readily obtained from titration data in 0.1 M NaCl by suitable extrapolation to $\alpha = 0$ when $\partial Fe/\partial\varkappa$,

TABLE VI

ELECTROPHORETIC MOBILITIES (μ) OF ACID POLYSACCHARIDES (5 mg/ml) AT 25°, 0.1 IONIC STRENGTH

*Type**	*pH***	a	μ (10^{-5} cm^2 volt^{-1} sec^{-1})		
			Asc.§	*Desc.*	*Desc. (calc.***)*
A	7.00	1.00	—32.6	—26.6	(—26.6)
B	7.00	1.00	—31.2	—26.4	(—26.4)
A	3.00	0.34	—26.4	—21.8	—17.9
B	3.00	0.14	—23.6	—19.3	—15.0
A	2.10	0.06	—21.1	—17.4	—14.1
B	2.10	0.02	—20.2	—16.5	—13.5

* Types A and B were, respectively, preparations CS13 and F3A.

** Buffers prepared according to MILLER AND GOLDER[15].

*** Descending mobilities are calculated from corresponding experimental values at pH 7 with the relation: $\mu = \mu'(1 + a)/2$ where μ' refers to values at pH 7.

§ Uncorrected.

and therefore ψ_0, becomes zero (eqn. (5)). The value of 3.20 found in this way is in good agreement with the pK_0 of glucuronic acid and with the value of 3.30 ± 0.08 obtained by use of eqns. (4) and (5). From a descending mobility of $18.0 \cdot 10^{-5}$ cm^2/V/sec at 25°, pH 7, $I = 0.1$, ψ_0 is calculated as $-1.22 \cdot 10^{-4}$ (e.s.u.), about 3 times greater than the value obtained from eqn. (5). However, use of the electrophoretic ψ_0 in eqn. (4) leads to an only slightly lower pK_0 of 3.10 ± 0.05. Consistent with a decreased ψ_0, the counter-ion binding for sodium hyaluronate may be estimated at 30 %, a value appreciably lower than that for CS-A under comparable ionic conditions.

Eqn. (4) is not valid when the electrostatic interaction between neighboring ionizable groups of a polymer is large enough to contribute to binding of counter-ions[17, 18]. It has been found that relative binding affinities for trivalent cations of the two polysaccharides, CS-A and CS-B, are similar to their relative binding affinities for protons[19]. It appears likely, therefore, that the observed differences in pK_0 are mainly a reflection of differences in interaction between carboxyl and sulfate groups.

CONCLUSION

The close similarity in polymer chain character of the isomeric polysaccharides indicates that the observed differences in solubility and in acidity of the carboxyl group are to be ascribed primarily to structural differences between the repeating units of the respective polysaccharides. This conclusion is confirmed by infra-red spectra which have already been published in a preliminary note[20]. While comparison of molecular constants and physical properties may be employed to differentiate CS-A and CS-C, spectral measurements furnish more easily applied and possibly more reliable criteria for this purpose.

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THE CROSS-β CONFIGURATION IN SUPERCONTRACTED PROTEINS

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SUMMARY

Models have been derived for the formation and lateral linkage of the transverse folds in the supercontracted state of the k-m-e-f group and other proteins. There are two solutions depending on whether the 180° bend is based on the so-called 2_7a fold or the 2_7b, and each requires certain sequences of amino-acid residues. Also discussed are the general features to be expected in X-ray diagrams, the elongation accompanying the transition from the cross-β to the parallel-β configuration, the "parallelism" or "anti-parallelism" of the aggregated chains, and the energetics of supercontraction.

INTRODUCTION

The property of supercontraction shown by the k-m-e-f group of fibrous proteins has long been thought of as a shortening of the α-configuration to some transversely-folded configuration[1], and this view eventually received its strongest support from the correlation of the phenomenon with the "cross-β" type of X-ray diffraction diagram[2], so called because the 4.65 Å reflection associated with the CO . . . HN linkage, which occurs on the equator in the "parallel-β" diagram produced by